

Université du Québec
Institut National de la Recherche Scientifique-
Institut Armand-Frappier

*Development of Cellulose Nanocrystal (CNC)
Reinforced Bio polymeric Matrix for
Microencapsulation of Bioactive Compounds*

Par

Tanzina Huq

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Jury d'évaluation

Président du jury et examinateur interne	Prof. Charles Ramassamy INRS-Institut Armand Frappier
Examineur externe	Prof. George Szatmari Université de Montreal
Examineur externe	Prof. Philippe Bébin Centre de Technologie Minérale et de Plasturgie
Directrice de recherche	Prof. Monique Lacroix INRS-Institut Armand Frappier
Co- directeur de recherche	Prof. Bernard Riedl Université Laval

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Résumé

La nanotechnologie a connu une croissance rapide en recherche, avec des applications en sciences alimentaires, comme une technologie émergente. Les potentiels de la nanotechnologie sont explorés dans divers projets de recherche pour la sécurité alimentaire et le développement d'aliments fonctionnels. La cellulose nanocristalline (CNC) est un nanomatériau cellulosique. En application dans les aliments, ce nanomatériau a été appliqué comme biopolymère alimentaire. Une matrice alginate de base renforcée de CNC a été développée pour la microencapsulation de composés bioactifs (probiotiques et les composés antimicrobiens). A un niveau de 5% en poids, la concentration en CNC a été optimisée dans une matrice d'alginate suite à l'amélioration de la résistance à la traction de 37% de films et la réduction de perméabilité à la vapeur d'eau de 31% par rapport à la matrice d'alginate. On a constaté qu'une concentration de 5% en poids dans une matrice d'alginate CNC a également amélioré les propriétés thermiques et morphologiques. Ce niveau optimisé de 5% en poids de CNC dans la matrice d'alginate a été utilisé pour la microencapsulation de composés antimicrobiens tels que la nisine et d'huiles essentielles afin d'améliorer la sécurité alimentaire des produits carnés prêts -à-manger (RTE) pendant le stockage, suite à une activité contre la bactérie *Listeria monocytogenes*. Il a été constaté que la nisine microencapsulée (63µg/ml) dans de l'alginate-CNC permet d'améliorer son activité et disponibilité 20 fois et a réduit la présence de *L. monocytogenes* par 3.04 log UFC / g par rapport à celle de la nisine libre (63µg/ml) pendant 28 jours de stockage à 4° C. Cette étude a révélé une nouveauté, soit par le développement de films d'alginate-CNC pour la microencapsulation de la nisine qui présentait un effet de protection des produits carnés prêts à consommer. Après avoir constaté l'effet antimicrobien de concentrations différentes de nisine micro-encapsulée et libre sur le système carné RTE, la plus faible concentration de nisine (16µg/ml) active a été évaluée en combinaison

avec l'origan 'compactum' ou la cinnamome 'cassia' (250 µg / ml) pour vérifier l'effet synergique de l'irradiation γ ou la pasteurisation à froid.

La popularité dans le monde des produits contenant des probiotiques a augmenté énormément dont certains qui sont très reconnus comme nutraceutiques. Mais ces produits montrent une viabilité très inférieure du probiotique pendant le stockage et en état gastrique. Notre matrice d'alginate-CNC développée a été utilisée pour la microencapsulation de probiotiques (*Lactobacillus rhamnosus* ATCC 9595) pour améliorer sa stabilité au cours du stockage. La concentration de CNC a été une fois de plus optimisée avec une matrice d'alginate en vérifiant la viabilité pendant la lyophilisation. Les résultats ont montré que 13 % en poids de CNC dans des microbilles d'alginate a seulement réduit la viabilité de *L. rhamnosus* par 0.01 log alors que le log de la viabilité des microbilles d'alginate a été réduit de 0.95 log après le séchage libre. On a également constaté que 13 % en poids de CNC en combinaison avec des microbilles d'alginate -lécithine a montré une plus grande amélioration de la viabilité au cours du stockage. Les résultats ont démontré que cette formulation a augmenté de 61 % la viabilité des probiotiques après 42 jours de stockage à 25° C par rapport à des contrôles de microbilles d'alginate. Cette formulation de microbilles d'alginate-CNC-lécithine a amélioré la viabilité de *L. rhamnosus* de 52 % au cours de la transition complète à travers le passage gastrique par rapport à microbilles d'alginate. Il y a eu aussi une nouvelle formulation de revêtements développée à l'aide d'alginate, de pectine et de CNC, afin d'améliorer la stabilité de *L. rhamnosus* à 25° C en utilisant un modèle de type 'composite central'. Cette formulation de revêtement a montré une bonne stabilité de *L. rhamnosus* à la température ambiante jusqu'à six mois de stockage. L'interaction observée entre les formulations de revêtement d'alginate-pectine-CNC a également présenté une viabilité de 84% après le passage gastrique.

En résumé, cette thèse a été entreprise pour développer des systèmes de microbilles biopolymères comestibles pour l'application dans le domaine de l'alimentation et à des fins nutraceutiques.

L'utilisation de CNC a créé une voie innovante pour l'industrie alimentaire et nutraceutique.

Tanzina Huq

Candidat Ph.D.

Prof. Monique Lacroix

Directrice de recherche

Abstract

Nanotechnology has been rapidly growing research in food application as an emerging technology. The potentials of nanotechnology have been explored in various food researches for food safety and development of functional foods. Cellulose Nanocrystals (CNC) are a cellulosic nanomaterial. In food applications, this nanomaterial has started its association with food biopolymers. A CNC reinforced alginate based matrix was developed for microencapsulation of bioactive compounds (probiotics and antimicrobials compounds). A 5 % wt CNC content was optimized in alginate based matrix with an improvement of tensile strength of 37% and a reduction of water vapour permeability of 31% compared to those of alginate matrix. It was found that 5 % wt CNC in alginate matrix also improved the thermal and morphological properties. This optimized 5 % wt CNC content in alginate matrix was applied for microencapsulation of antimicrobial compounds such as nisin and essential oils in order to improve the food safety of ready-to-eat (RTE) meat products during storage against *Listeria monocytogenes*. It was found that microencapsulated nisin (63µg/ml) in alginate-CNC beads improved the activity of nisin 20 times and reduced the counts of *L. monocytogenes* by 3.04 log CFU/g compared to those of free nisin (63µg/ml) during 28 days of storage at 4° C. This study revealed a novelty by the development of alginate-CNC beads for microencapsulation of nisin that exhibited protection effect of RTE meat products. After obtention of a antimicrobial effect of different concentrations of microencapsulated and free nisin on RTE meat system, the lowest concentration of nisin (16µg/ml) was evaluated in combination with *Origanum Compactum* or *Cinnamomum Cassia* (250 µg/ml) to check the synergistic effect of γ -irradiation or cold pasteurization.

The worldwide popularity of probiotic containing products have been increasing tremendously and these products are known as nutraceuticals. But the viability of probiotics in these products is much

lower during storage and gastric conditions. Our alginate-CNC matrix was used for microencapsulation of probiotics (*Lactobacillus rhamnosus* ATCC 9595) to improve its stability during storage. The CNC concentration was once more optimized with an alginate matrix by checking the viability during freeze drying. Results demonstrated that 13 wt% of CNC in alginate microbeads only reduced the viability of *L. rhamnosus* by 0.01 log whereas alginate microbead reduced the viability by 0.95 log after freeze drying. It was also found that 13 wt% of CNC in combination with alginate-lecithin microbead showed more improvement of viability during storage. Results showed this formulation increased the probiotic viability by 61% after 42 days of storage at 25°C compared to control alginate microbead. This alginate-CNC-lecithin microbeads formulation improved the viability of *L. rhamnosus* by 52% during complete transition through the gastric passage as compared to alginate microbead. There was also a novel coating formulation developed by using alginate, pectin and CNC in order to improve the stability of *L. rhamnosus* at 25°C by using central composite experimental design. This coating formulation showed a good stability of *L. rhamnosus* at room temperature up to six months of storage. The interaction found between alginate-pectin-CNC coating formulations exhibited a viability of 84% after complete transition through the gastric passage.

In summary, this thesis was undertaken to develop biopolymer edible microbeads for food applications and nutraceutical purposes. The utilization of CNC established an innovative route for the food and nutraceutical industry.

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List of Abbreviations

Analysis of variance (ANOVA)	Hydroxypropyl methyl cellulose (HPMC)
Brain Heart Infusion (BHI)	International Atomic Energy Agency (IAEA)
Chloramphenicol (CAM)	Lactic acid bacteria (LAB)
Carboxymethyl cellulose (CMC)	Modified atmospheric packaging (MAP)
Cellulose acetate phthalate (CAP)	Natural Sciences and Engineering Research Council of Canada (NSERC)
Cellulose nanocrystals (CNC)	Puncture deformation (PD)
Central composite design (CCD)	Puncture strength (PS)
Cetyl trimethyl ammonium bromide (CTAB)	Ready-to-eat (RTE)
Code of Federal Regulations (CFR)	Response surface methodology (RSM)
Colorectal cancer (CRC)	Scanning Electron Microscopy (SEM)
Differential Scanning Calorimetric (DSC)	Simulated gastric fluid (SGF)
Elongation at break (Eb%)	simulated intestinal fluid (SIF)
Essential Oils (EOs)	Tensile modulus (TM)
Facility Electron Microscopy Research (FEMR)	Tensile strength (TS)
Food and Agriculture Organization (FAO)	Thermo Gravimetric Analysis (TGA)
Food and Drug Administration (FDA)	Viscoelasticity (Y)
Fourier Transform Infrared (FTIR)	Water vapor permeability (WVP)
Generally recognised as safe (GRAS)	Water-in-oil (W/O)
Health Products and Food Branch (HPB)	Whey Protein Isolate (WPI)
Hydroxyl propyl methylcellulose acetate succinate (HPMCAS)	World Health Organization (WHO)
	X-ray Diffraction (XRD)

Introduction

Current interest in nanotechnologies has led to the application of Cellulose Nanocrystals (CNC) in food application. In today's world, Canada is the world's leading producer of CNC and ranks first in research in this area. The North American market for CNC may reach \$250 million according to Canada's forest industry news. The overall range of material applications for CNC is virtually limitless; in addition, the possibility of controlling their biosynthetic pathways opens up new windows to encouraging their use as a facile energy resource for food application. CNC has been found to be an innovative nanomaterial due to its good reinforcing properties with biopolymers over the past 20 years (Samir *et al.*, 2005). Among the biopolymers, alginate is one of the most widely used biopolymers as a bioactive coating in food application. It has a very good applicability to prepare microcapsules owing to its good biocompatibility, biodegradability, non-toxicity, gelation and mucoadhesion properties (Lertsutthiwong *et al.*, 2008), with the addition of some other additives.

Bioactive compounds (antimicrobial and probiotic) are of growing world wide interest due to the consumer demand on food safety and nutritional aspects for human health. Antimicrobials are used to reduce pathogenic bacteria such as *Listeria monocytogenes* in the food supply. Bacteria such as *L. monocytogenes* causes 94% hospitalization which results in a 15.6% death rate in the US each year (Scallan *et al.*, 2011). In 2008, there was a listeriosis outbreak in Canada and 22 people died due to the contamination of Maple Leaf Food products (meat products) by *L. monocytogenes*. Thus it is important to add antimicrobials in food for health safety. The direct addition of antimicrobial compounds in food system showed degradation by interacting with individual food components. On the other hand, probiotics are living microorganism food ingredients that have a beneficial effect on human health. Lactic acid bacteria used as probiotics are commonly incorporated into

foods to provide a wide variety of health benefits but stabilization of probiotics at room temperature and under gastric environment is a challenging endeavour. However, microencapsulation has been found to be a powerful technology to protect the antimicrobials and probiotics from these adverse conditions. This technology is based on the proper utilization of biopolymers which are normally referred to as bioactive coatings. The aim of this research was to develop CNC reinforced alginate matrix for microencapsulation of antimicrobials and probiotics.

Chapter -1
Literature Review

1.1 Introduction

This literature review provides the information about microencapsulation of bioactive compounds in biopolymeric matrix. The importance of different biopolymer matrixes or matrices described along with different microencapsulation processes. CNC is a new bio-nanomaterial which delivers interesting properties with biopolymer matrix. The part of this literature review has been published in *Critical Reviews of Food Science and Nutrition*. The text is reproduced from the following literature review with the addition of antimicrobial compounds, some more information about biopolymers and microencapsulation:

Huq, T., Khan, A., Khan, R.A., Riedl, B. & Lacroix, M., (2013). Encapsulation of Probiotic Bacteria in Biopolymeric System. *Critical Reviews in Food Science and Nutrition*, 53:909–916.

The pulished literature review was prepared by Tanzina Huq with continuous direction of my director Prof. Monique Lacroix. Prof. Bernard Riedl, my co-director, helped me to correct and improve the draft. Avik Khan has helped me to write the microencapsulation technology. Dr. Ruhul A. Khan has corrected the draft.

1.2 Bioactive compounds

The range of food components that could be considered as bioactive compounds include vitamins, minerals, functional lipids, probiotics, amino acids, peptides and proteins, fatty acids and antioxidants/ antimicrobials. Among the bioactive compounds, antimicrobials and probiotics have been chosen due to their potential activity for the food industry. Antimicrobial compounds are used for food safety, probiotics and for nutraceutical applications. Both antimicrobials and probiotics are two important classes of bioactive compounds that need to be protected from adverse condition as antimicrobials can readily react with food components and probiotics are very sensitive to oxygen. Our present review involved essential oils and bacteriocins as antimicrobial compounds.

1.2.1 Essential Oils (EOs)

EOs are volatile, natural, complex compounds characterized by a strong odour and are formed by aromatic plants as secondary metabolites. They are usually obtained by steam or hydro-distillation. It has long been recognized that EOs have antimicrobial properties for food preservation. Besides antimicrobial properties, EOs or their components have been shown to exhibit antiviral, antimycotic, antitoxigenic, antiparasitic, and insecticidal properties. They are gaining interest for their potential as preservative ingredients or decontaminating treatments, as they have GRAS status and a wide acceptance from consumers (Burt and Reinders, 2003; Burt, 2004). The antimicrobial components that are commonly found in the essential oil fractions have a wide spectrum of antimicrobial activity, with potential for control of *L. monocytogenes* and spoilage bacteria within food system (Oussalah *et al.*, 2007). Table-1 represents the major components of some EOs with antimicrobial properties.

1.2.1.1 Uses of EOs in Food Preservation

The potential application of EOs as natural antimicrobials and antioxidants has been found in meat, fish, fruit, vegetables and dairy products. Eugenol and coriander, clove, oregano and thyme oils were found to be effective at levels of 5-20 µl/g by inhibiting the growth of *L. monocytogenes* in meat products (Burt, 2004). Some packaging technologies such as vacuum, modified atmospheric packaging (MAP) have been used to enhance the antimicrobial activity of EOs on food systems. In general, MAP is a food packaging technology in which the proportion of oxygen, carbon dioxide and nitrogen are maintained in a sealed food product. Chouliara *et al.* (2007) investigated the combined effect of oregano oil and MAP on the shelf-life extension of fresh chicken meat stored at 4°C. Valero *et al.* (2006) declared that the combination of MAP and eugenol or thymol was an interesting tool to preserve the quality, safety and functional properties of grapes. Dussault *et al.* (2014) showed that oregano (500 ppm) reduced around 2 log CFU/g growth of *L. monocytogenes* in vacuum packed RTE cooked ham compared to control ham.

1.2.1.2 Major Components of EOs

Table 1.1: The major components of some EOs with antimicrobial properties are presented:

Common name of EO	Latin name of plant sources	Major components	Approximate concentration of major components of EOs (%)	Antimicrobial against the bacteria	References
Oregano	<i>Origanum Compactum</i>	Carvacrol Thymol	Trace-80% Trace-64%	<i>Listeria monocytogenes</i> <i>Escherichia coli</i>	Daferera <i>et al.</i> (2000); Turgis <i>et al.</i> (2012); Daferera <i>et al.</i> (2003).
Cinnamon	<i>Cinnamomum Cassia</i>	Trans-cinnamaldehyde	65%	<i>L. monocytogenes</i> <i>E.coli</i> <i>Salmonella typhimurium</i>	Caillet <i>et al.</i> (2006); Turgis <i>et al.</i> (2012).
Thyme	<i>Thymus vulgaris</i>	Thymol Carvacrol γ -Terpinene p- Cymene	10-64% 2-11% 2-31% 10-56%	<i>L.monocytogenes</i> <i>E. coli</i>	Turgis <i>et al.</i> (2012); Daferera <i>et al.</i> (2000).
Rosemary	<i>Rosmarinus officinalis</i>	Camphor 1,8-cineol	2-14% 3-89%	<i>L. monocytogenes</i> <i>E.coli</i> <i>Staphylococcus aureus</i>	Daferera <i>et al.</i> (2003); Dussault <i>et al.</i> (2014).

1.2.2 Bacteriocin: Nisin Antimicrobial Polypeptide

Bacteriocins are defined as a protein-based substances possessing antimicrobial activities produced during the growth of Gram (+) and Gram negative (-) bacteria. The first known bacteriocin was identified in *Escherichia coli* as an antimicrobial protein and named colicin. Bacteriocins are often confused with antibiotics, however the main differences is that antibiotics are not ribosomally synthesized, while they also differentiate from antibiotics on the basic mode of action, antimicrobial spectrum, toxicity and resistance mechanisms (Cleveland *et al.*, 2001).

The interest in bacteriocins produced by GRAS (generally recognized as safe) microorganisms has been leading to considerable interest for nisin, being the first bacteriocin to gain widespread commercial application since 1969 after the approval from FAO/WHO (Food and Agriculture Organization/World Health Organization) (De Arauz *et al.*, 2009). Nisin has been approved by the Food and Drug Administration (FDA) since 1988, satisfying the demands for natural foods with less chemical additives (Penna *et al.*, 2005). It is a natural antimicrobial peptide produced by strains of *Lactococcus lactis* subsp. *lactis* that effectively inhibits Gram (+) bacteria and the outgrowth of spores of Bacilli and Clostridia (Balciunas *et al.*, 2013). Nisin could also be effective against Gram (-) bacteria after having gone through some modification (Stevens *et al.*, 1991). Structurally, it is a 34 amino acid polypeptide, presenting cationic and hydrophobic characteristics, with a molar mass of 3500 Da, belongs to the lantibiotic family as it contains lanthionine and methyl lanthionine groups. In addition, the unusual amino acids composition might be responsible for the important functional properties of nisin, i.e. acid tolerance, thermo stability at low pH and a specific bactericidal mode of action (De Vuyst and Vandamme, 1992). The solubility, stability and biological activity of nisin are dependent on the pH of the solution. In fermentation process, at pH < 6.0, more than 80% of nisin produced is released into the medium whereas at pH > 6.0, most of

the nisin is associated with the cellular membrane, but not the cytoplasm. Solubility and stability increase drastically with the lowering of pH and it was found that nisin is stable at pH 2.0. At this pH, nisin can be autoclaved at 121° C for 15 min without inactivation, while, on the other hand, in neutral and alkaline conditions nisin is almost insoluble (Penna *et al.*, 2005).

1.2.2.1 Application of Nisin in Food as a Natural Antimicrobial

Nowadays, consumers prefer minimally processed foods of high quality, prepared without artificial preservatives, safe and with long shelf-life. Furthermore, nisin is the only bacteriocin that has been officially employed in the food industry and its use has been approved world-wide (Cleveland *et al.*, 2001). There are numerous applications of nisin as a natural food preservative, including dairy products, canned foods, processed cheese and meat products. The FAO/WHO Codex Committee on milk and milk products accepted nisin as a food additive for processed cheese at a concentration of 12.5 mg pure nisin per kilogram product (De Arauz *et al.*, 2009). Table-2 shows the world wide food application of nisin. The meat system is also one of the best examples for the use of nisin. Nitrates are commonly used to prevent clostridial growth in meat; however, safety concerns regarding the presence of nitrites have prompted the food industry to look for alternative methods of preservation. Nisin or its combination with lower levels of nitrate can prevent the growth of *Clostridium* (Rayman *et al.*, 1983; Cleveland *et al.*, 2001). During processing, this ready-to-eat (RTE) food products can readily be contaminated by pathogenic bacteria (*L. monocytogenes*). Nisin showed very strong antimicrobial effect in cooked ham against *L. monocytogenes* (Marcos *et al.*, 2007).

Table-1.2: Application of nisin in world*

Country	Food in which nisin is permitted	Maximum Level (IU/g)
Argentina	Processed Cheese	500
Australia	Cheese, processed cheese, canned tomatoes	No limit
Belgium	Cheese	100
Brazil	Cheese, canned vegetables, sausages, meat products	500
France	Processed Cheese	No limit
Italy	Cheese	500
Mexico	Permitted additive	500
Netherlands	Factory cheese, processed cheese, cheese powder	800
Peru	Permitted additive	No limit
Russia	Dietetic processed cheese, canned vegetables	8000
USA	Pasteurized processed cheese spread, meat products	10,000
UK	Cheese, canned foods, clotted cream	No limit

*Source: Adapted from De Arauz *et al.* (2009).

1.2.3 Probiotics

People have been using LAB for more than 4000 years for foods' fermentation. Today, probiotics are also used in a variety of fermented dairy products and their manufacture involves fermentation: microbial process by which lactose is converted into lactic acid. Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) define probiotics as “Live microorganisms (bacteria or yeasts), which when ingested or locally applied in sufficient numbers confer one or more specified demonstrated health benefits for the host”(FAO/WHO, 2001). LABs are the most important probiotic microorganisms typically associated with the human gastrointestinal tract. These bacteria are gram-positive, rod-shaped, non-spore-forming, catalase-negative organisms that are devoid of cytochromes and are of non-aerobic habit but are aero-tolerant, acid-tolerant and strictly fermentative; lactic acid is the major end-product of sugar fermentation. A few of the known LABs that are used as probiotics are *Lactobacillus acidophilus*, *Lactobacillus amylovorus*, *Lactobacillus casei*, *Lactobacillus crispatus*, *Lactobacillus delbrueckii*, *Lactobacillus gasseri*, *Lactobacillus johnsonii*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus* etc. (Anal and Singh, 2007). Lactobacilli, as a part of the commensal microbial flora of humans and mammals and main representatives of the probiotic bacteria, might be useful candidates in prevention and treatment of infections caused by multi-resistant bacteria due to their ability to modulate the immune responses of the host and to protect the host from pathogens by competitive exclusion (Brachkova *et al.*, 2010). Other common probiotic microorganisms are the bifidobacteria. Bifidobacteria are also gram-positive and rod-shaped but are strictly anaerobic. These bacteria can grow at pH in the range 4.5-8.5. Bifidobacteria actively ferment carbohydrates, producing mainly acetic acid and lactic acid in a molar ratio of 3:2 (v/v), but not carbon dioxide, butyric acid or

propionic acid. The most recognized species of bifidobacteria that are used as probiotic organisms are *Bifidobacterium adolescentis*, *Bifidobacterium animalis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium infantis*, *Bifidobacterium lactis* and *Bifidobacterium longum*. Other than these bacteria, *Bacillus cereus* var. *toyoi*, *Escherichia coli* strain nissle, *Propionibacterium freudenreichii*, and some types of yeasts, e.g. *Saccharomyces cerevisiae* and *Saccharomyces boulardii* have also been identified as having probiotic effects (Holzapfel *et al.*, 2001). Some importance effect of probiotic are discussed below.

1.2.3.1 Intestinal Tract Health

A number of studies have found probiotic consumption to be useful in the treatment of many types of diarrhoea, including antibiotic-associated diarrhoea in adults, travellers' diarrhoea, and diarrhoeal diseases in young children caused by rotaviruses. The most commonly studied probiotic species in these studies have been *Lactobacillus GG*, *L. casei*, *B. bifidum* and *S. thermophilus*. Because diarrhoea is a major cause of infant death worldwide and can be incapacitating in adults, the widespread use of probiotics could be an important, non-invasive means to prevent and treat these diseases, particularly in developing countries. Probiotic bacteria have also been shown to preserve intestinal integrity and mediate the effects of inflammatory bowel diseases, irritable bowel syndrome, colitis, and alcoholic liver disease. In addition, LAB may improve intestinal mobility and relieve constipation (Pitino *et al.*, 2010; Isolauri *et al.*, 1991; Nanji *et al.*, 1994).

1.2.3.2 Nutrient Synthesis and Bioavailability

Fermentation of food with LAB has been shown to increase the folic acid content of yogurt, bifidus milk and kefir and to increase niacin and riboflavin levels in yogurt, vitamin B₁₂ in cottage cheese and vitamin B₆ in cheddar cheese. In addition to nutrient synthesis, probiotics may improve the digestibility of some dietary nutrients such as protein and fat. Short-chain fatty acids such as lactic

acid, propionic acid and butyric acid produced by lactic acid bacteria may help maintain an appropriate pH and protect against pathological changes in the colonic mucosa (Chen and Subirade, 2009; Kruis *et al.*, 1997).

1.2.3.3 Probiotic Antimicrobial Activity

The importance of probiotics in human nutrition has been gaining recognition in recent years. Marianelli *et al.* (2010) proposed an improved *in vitro* model for the study of probiotic antimicrobial activity against enteropathogens, by attempting to re-create, in a common culture medium, environmental growth conditions comparable to those present in the small intestine. In that study, a preliminary experiment was carried out in order to find out a culture medium able to support both probiotics and pathogens. This experiment was done with the aim of obtaining correct assessment of the interaction under shared growth conditions. Brain Heart Infusion (BHI) medium was selected as the common culture medium and was therefore used in antimicrobial activity assays. The interactions between *Salmonella* 1344 and *Lactobacillus rhamnosus-Lactobacillus reuteri* were then assessed at different pH and oxygen availability conditions mimicking the small intestinal environment. *L. rhamnosus GG* ATCC 53103 had the strongest antimicrobial effect, in particular under anaerobic conditions and at lower pH levels. Its antagonistic activity involved both lactic acid and secreted non lactic acid molecules.

1.2.3.4 Probiotics for Cancer Prevention

Studies on the effect of probiotic consumption on cancer appear promising. Colorectal cancer (CRC) is the biggest cause of death from cancer in the Western world. Approximately 70% of CRC is associated with environmental factors most likely due to the diet. The fermented milk containing probiotic cultures can play a protective role against CRC. Interventional studies have shown a shift of intermediate markers of CRC risk in human subjects from a high to low risk

pattern after ingestion of fermented milks or probiotics. Animal studies consistently show a reduction in chemically induced colorectal tumor incidence and aberrant crypt formation accompanying probiotic administration. *In vitro* studies also provide evidence of protection, and permit a better understanding of active compounds involved, and of the mechanisms underlying their anti-carcinogenic effects. Probiotics may beneficially modulate several major intestinal functions: detoxification, colonic fermentation, transit, and immune status, which may accompany the development of colon cancer (Saikali *et al.*, 2004; Hirayama and Rafter, 2000). LAB or a soluble compound produced by the bacteria may interact directly with tumour cells in culture and inhibit their growth. LAB significantly reduced the growth and viability of the human colon cancer cell line HT-29 in culture, and di-peptidyl peptidase IV and brush border enzymes were significantly increased, suggesting that these cells may have entered a differentiation process (Baricault *et al.*, 1995).

1.3 Microencapsulation of Bioactive Compounds

Microencapsulation has been defined as the technology of packaging solid, liquid and gaseous materials in small capsules that release their contents at controlled rates over prolonged periods of time. A microcapsule consists of a semi permeable, spherical, thin and strong membrane surrounding a solid or liquid core, with a diameter varying from a few microns to 1 mm. It can be used for many applications in the food industry, including stabilizing the core material, controlling the oxidative reaction, providing sustained or controlled release (both temporal and time-controlled release), masking flavours, colours or odours, extending the shelf life and protecting components against nutritional loss (Champagne and Fustier, 2007). Microencapsulation can reduce the volatility of EOs and also protect the EOs from in contact with the fat components of food (Burt, 2004). The inactivation of nisin has been enhanced by the interaction with proteolytic

enzymes of food (meat products). Only microencapsulation can protect nisin from this inactivation during storage (Hosseini *et al.*, 2014). Similarly, for enhancing the viability of probiotic, microencapsulation facilitates handling of cells and allows a controlled dosage. Food-grade polymers such as alginate, chitosan, carboxymethyl cellulose (CMC), hydroxypropyl methyl cellulose (HPMC), carrageenan, gelatin and pectin are mainly applied, using various encapsulation technologies (Anal and Singh, 2007).

Some of the most common microencapsulation technologies are described below.

1.3.1 Extrusion

Extrusion is a widely used technology that produces a small droplet of an encapsulation material by forcing the solution through nozzles or small openings in droplet-generating devices. The smaller the inner diameter of the nozzle or openings, the smaller will be the capsules. Industry-oriented groups quite often have the incorrect assumption that this procedure does not allow for large scale production and is only suitable for laboratory scale processes. However, enormous advances have been made in the up-scaling of encapsulation processes using extrusion technology. Large scale droplet production can be achieved by multiple-nozzle systems, spinning disc atomizer, or by jet-cutter techniques (Kailasapathy, 2002). Extrusion is mainly utilized for probiotic encapsulation. This method is a simple and cheap method with gentle operations which makes cell injuries minimal and causes relatively high viability of probiotic cells. Biocompatibility and flexibility are some of the other specifications of this method. A hydrocolloid solution is first prepared, probiotics are added and the solution is dripped through a syringe needle or nozzle. The droplets are allowed to fall into a hardening solution. In this technique, alginate, *k*-carrageenan, *k*-carrageenan plus locust bean gum, xanthan gum plus gellan, alginate plus corn starch and whey proteins have been used as wall materials for encapsulation of lactobacilli and bifidobacteria. The

size of the microcapsules is affected by the nozzle size. The diameter of the obtained alginate beads is also increased as the concentration of sodium alginate increases, but the alginate concentration does not significantly influence the numbers of free cells. A mixture of gellan and xanthan gum has better technological properties than *k*-carrageenan, or locust bean gums, but the shape and size of the gellan and xanthan gum capsules has been found to vary (Rokka and Rantamaki, 2010).

1.3.2 Emulsification

Emulsification is a very successful technique for both antimicrobial and probiotic microencapsulation. Contrary to the extrusion technique, it can be easily scaled up and the diameter of produced beads is considerably smaller (25 µm-2 mm). However, this method requires more cost for performance compared with the extrusion method due to need of using vegetable oil for emulsion formation. McClements, *et al.* (2009) recently reviewed and discussed all the interactions that have to be taken into account when designing an encapsulation system by emulsification for bioactive food molecules. Emulsification is defined as a process of dispersing one liquid in a second immiscible liquid. By including the core material in the first liquid we can encapsulate the bioactive component. In most cases researchers and companies choose to encapsulate the bioactive molecules in food grade (GRAS) derived molecules by applying electrostatic interactions, hydrophobic interactions, or hydrogen bonding between the bioactive molecule and an encapsulating molecule. In this technique, a small volume of bioactive/polymer slurry (as a dispersed phase) is added to the large volume of vegetable oil (as a continuous phase) such as soy-, sun flower-, corn-, millet or light paraffin oil. Resulting solution becomes quite homogeneous by proper stirring/agitating, in water-in-oil (W/O) emulsion forms. Once W/O emulsion forms, the water soluble polymer such as alginate, pectin becomes insoluble by means of cross linking agent (calcium chloride) and thus makes gel particles in the oil phase. Using

emulsifiers causes formation of beads with smaller diameters, because these components decrease interfacial tension of the water and oil phases. It has been claimed that by applying emulsifiers like Tween 80 and lauryl sulphate together, beads with a range of 25-35 μ m in diameter can be produced. In the emulsion technique relevant to alginate, a fat soluble acid such as acetic acid is usually added to the encapsulation mixture. Thereby, pH of alginate solution is reduced to approximately 6.5, at which the gelation process of alginate with calcium ions starts. After gel formation, the encapsulated mixture is poured into water to separate the oil phase by decantation. It has been reported that concentration and viscosity of the encapsulation mix before gelation and its agitation rate are the main parameters that control the diameter of the final formed microbeads. It should be noted that the bead diameter, apart from having a crucial effect on the viability of probiotic cells, affects the metabolic rate and sensory properties of the final product, and also affects the distribution and dispersion quality of the microbeads within the product (Krasaekoopt *et al.*, 2003; Picot and Lacroix, 2004).

1.3.3 Drying Methods

Drying of the encapsulated mixture in order to produce cell powders/granules can be achieved by different methods. The most important of these methods are freeze drying, spray drying and fluidized bed drying. Typical survival rates in the spray-drying and freeze drying processes are in the range of 70–85%. Although such a survival rate may be acceptable, the prolonged storage stability of the product is often low. The presence of deoxidants and desiccants has been found to improve the cell survival. In general, the drying process causes some injuries to the microbeads, release of some cells and reduces viability of the cells.

1.3.3.1 Spray Drying

Spray drying is a one of the most commonly used microencapsulation technologies. It is being applied for both bioactive molecules and living probiotics. It is a fast and relatively cheap procedure that, when adequately performed, is highly reproducible. The principle of spray drying is dissolving the core in a dispersion of a chosen matrix or shell material. Figure 1.1 represents the spray drying method. The dispersion is subsequently atomized in heated air. This promotes fast removal of the solvent (water). The powdered particles are then separated from the drying air at the cyclone outlet at a lower temperature. The relative ease and also the low cost are the main reasons for the broad application of spray drying in industrial settings. This technique represents problems when utilized for probiotic encapsulation where the bacteria may leak with the product during hydration. It has been reported that bifidobacteria are very sensitive to high inlet temperatures (O'Riordan *et al.*, 2001). Temperatures above 60° C interfered with survival of the bacteria and also influenced the spray drying process as sticky products were reported with this type of bacteria in the cyclone (Kailasapathy, 2002). Thus it would be advisable to investigate the sensitivity of a probiotic for increased temperatures before proposing spray drying as the technology of preference for a specific bacterium. But spray drying technology has many advantages over the other technologies for other bioactive food components such as EOs, bacteriocins, vitamins, minerals, flavors, unsaturated oils, and enzymes. Only water-based dispersions are applied in spray drying. Therefore the matrix should have a high solubility in water. In most instances hydrophilic carbohydrate molecules are applied. These carbohydrates undergo a transition to a so-called glass (i.e., an amorphous solid) when the dispersion is rapidly evaporated. Usually the product is very stable and allows for a significant increase in shelf life (Augustin and Hemar, 2009).

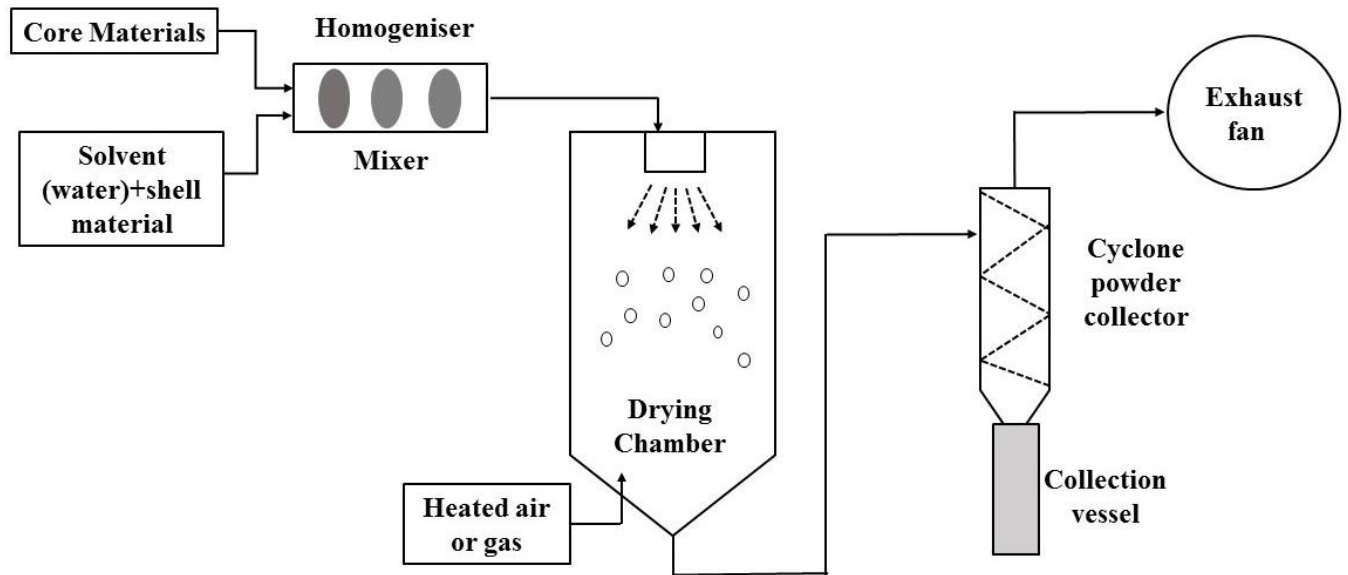


Figure 1.1: Schematic diagram of spray drying method.

1.3.3.2 Fluid Bed Drying

An improved spray dry technology that expands the field of application is the fluid bed coating methodology. In this technology, the bioactive food components are suspended in air and the matrix molecules are sprayed onto the bioactive components. This forms a capsule (Champagne and Fustier, 2007). The choice for matrix molecules is broader than for traditional spray drying. It may be fats, proteins, carbohydrates but also emulsifiers. Also it is useful for applying an additional layer of molecules for controlled release of bioactives during storage. In principle of fluid bed drying, the core materials should be always solid compounds. For example, this technology would be efficient for bacteriocin but not for volatile compounds like essential oils (De Vos *et al.*, 2010).

1.3.3.3 Freeze Drying

Freeze drying in combination with matrix molecules has been proposed as an alternative for spray drying of heat sensitive bacteria. A schematic representation of freeze drying method is presented in Figure 1.2. However, drying in general should not be considered to be a very efficacious methodology for preservation of bioactivity of living cells. Probiotics are exposed to damage from the process conditions such as very low freezing temperatures and dehydration. Cells are first frozen to below the critical temperature of the formulation, and then dried by sublimation under high vacuum in two phases: primary drying, during which unbound water is removed and secondary drying, during which the bound water is removed. These stages can damage the constituents of the cell wall and lead to cell death (Jalali *et al.*, 2012). In the freeze drying technique, heat injuries to the cells are minimal compared with other techniques. Also, cryo protectants must be used to inhibit cold injuries to the cells (Augustin and Hemar, 2009) and microencapsulation using biopolymers can also protect the cells during drying (Chana *et al.*, 2011). Figure 1.3 a) and b) represent the images of microencapsulated bioactives in alginate-CNC microbeads before and after freeze drying, respectively.

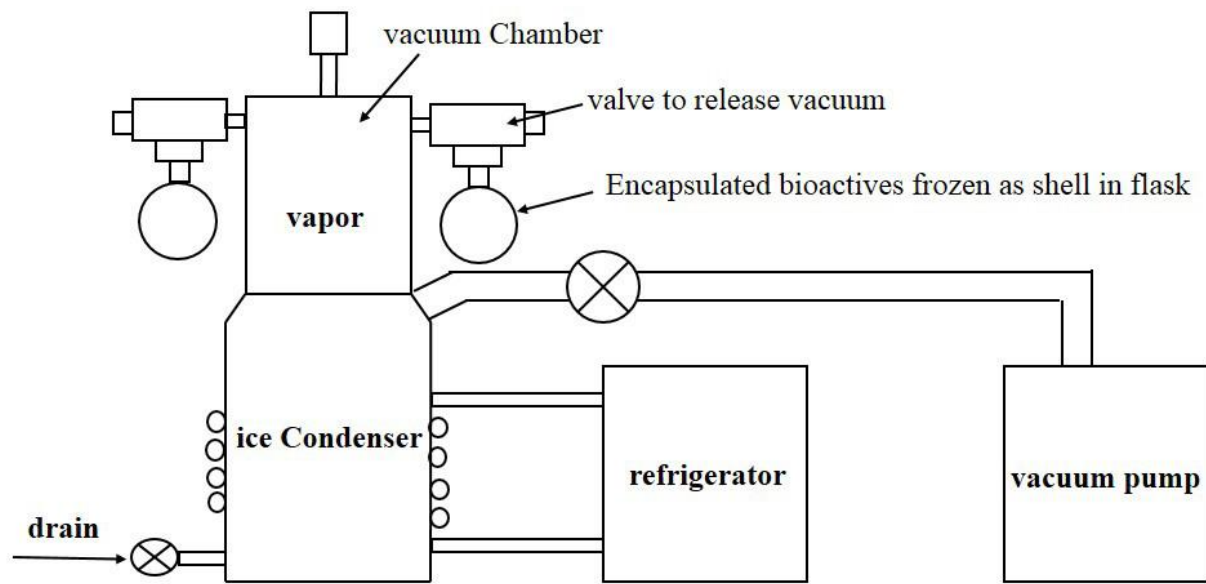


Figure 1.2: Freeze drying method

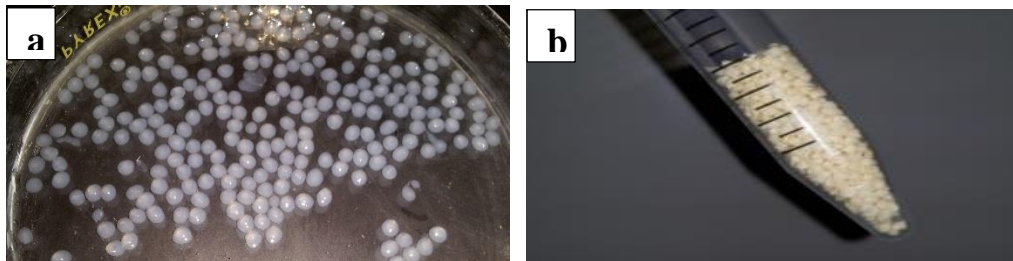


Figure 1.3: Microencapsulated bioactives a) before freeze drying and b) after freeze drying.

1.3.4 Compression Method

The compression coating method has been found as a novel encapsulation technique for improving the viability of living cells during storage and gastric treatment. Biopolymers (sodium alginate, pectin, gellan gum) which can form gels after being hydrated, have been exploited as the prime coating material. Probiotic containing powders are first compressed into a pellet, which is then encapsulated with the coating material by further compression. Chan and Zhang, (2002)

demonstrated this technology for probiotic encapsulation to keep its viability stable during gastric transition.

1.4 Biopolymers Used for the Microencapsulation of Bioactive Compounds

1.4.1 Microencapsulation in Alginate System

One of the most commonly applied polysaccharides is alginate. Alginates are linear polymers with 1-4 linked- β -D mannuronic acid (M) and α -L-guluronic acid (G) residues arranged as blocks of either type of unit or as a random distribution of each type. They can be obtained in different G/M ratios which provides different degrees of mechanical stability. Alginate is very compatible with most of the encapsulation technologies. Conventional extrusion method has been used to microencapsulate both antimicrobial and probiotic where sodium alginate is extruded in calcium chloride (CaCl_2) solution. Anal and Singh, (2007) reported that probiotics encapsulated in calcium-alginate beads are better protected, as shown by an increase in the survival of bacteria under gastric conditions, compared to the non-encapsulated state. The authors indicated that the viability of encapsulated bacteria in gastric fluid increased with an increase in capsule size. However, it was reported that very large calcium alginate beads (>1 mm) caused a coarseness of texture in live microbial feed supplements and that small beads of size less than 100 μm in diameter do not significantly protect the bacteria in SGF, compared with free cells. These studies indicate that these bacteria should be encapsulated within a particular size range (Anal *et al.*, 2003). In this work, alginate microbeads (20-70 μm) were prepared by the mixture of probiotic cells and sodium alginate in vegetable oil and subsequently cross-linked with CaCl_2 . It was found that the probiotic loaded alginate microparticles remained stable during storage at 4°C in 0.05 M CaCl_2 and in milk (2% fat), sour cream and yogurt for up to 16 days and in SGF (pH 2.0) for 1 h at 37°C. They also showed that *B. bifidum* survived in higher numbers in frozen milk in beads made from alginate

than in beads made from *k*-carrageenan (Hansen *et al.*, 2002). Santa-Maria *et al.* (2012) used the spray dry technology for gelation of alginate with CaCl₂ to prolong the stability of bioactive. Research has been conducted on encapsulation of nisin in alginate microbead prepared by extrusion method and applied on real food system. Results demonstrated that encapsulated nisin had better stability in food system compared to non-encapsulated one (Millette *et al.*, 2007). Soliman *et al.*, (2013) reported an emulsification-extrusion method for microencapsulation of EOs and found that the microencapsulated EOs showed 50% antimicrobial activity after 8 days of storage.

1.4.2 Microencapsulation in Gelatin and Polysaccharide System

Gelatin is useful as a thermally reversible gelling agent for encapsulation. Due to its amphoteric nature and positive charge (which is dependant on pH), it is also an excellent candidate for incorporating with anionic gel forming polysaccharides, such as alginate, pectin and gellan gum. These hydrocolloids are miscible at pH > 6, because the net charge of gelatin becomes positive when the pH is adjusted below its isoelectric point and causes a strong interaction with the negatively charged polysaccharides (Annan *et al.*, 2008). Guerin *et al.* 2003 reported a mixed gel encapsulating matrix composed of alginate, pectin and whey proteins for encapsulation of *Bifidobacterium* cells. Authors investigated the protective effects of gel beads without extra membrane and gel beads coated with extra membranes, formed by the conjugation of whey protein and pectin, in simulated gastric pH and bile salt solutions on the survival of free and encapsulated *B. bifidum*. After 1 h of incubation in acidic solution (pH 2.5), the free cell counts decreased by 4.75 log, compared with a decrease of < 1 log for entrapped cells. The free cells did not survive after 2 h of incubation at pH 2.5, whereas the immobilized cells decreased by about only 2 log. After incubation (1 or 3 h) in 2 and 4% bile salt solutions, the mortality for *B. bifidum* cells in

membrane-free gel beads (4-7 log) was greater than that for free cells (2-3 log). However, the counts of cells immobilized in membrane coated gel beads decreased by < 2 log. The double membrane coating enhanced the resistance of the cells to acidic conditions and higher bile salt concentrations. Singh *et al.* (2014) developed a gelatin-polysaccharide matrix (e.g. maltodextrin, dextran and sodium carboxymethyl cellulose) for antimicrobial delivery at vaginal application. Metronidazole drug was encapsulated in this matrix and used against *E. coli* and *B. subtilis*. Chen *et al.* (2010) also demonstrated the antimicrobial activity of gelatin matrix containing aloe gel against *E. coli*, *S. aureus* and *B. cereus*.

1.4.3 Chitosan-coated Alginate Encapsulate System

Chitin is a homopolymer comprised only of 2-acetamido-2-deoxy- β -D-glucopyranose residues, whereas chitosan is a heteropolymer mainly composed of 2-amino-2-deoxy- β -D-glucopyranose repeating units but still retaining a small amount of 2-acetamido-2-deoxy- β -D-glucopyranose residues. Chitin is the second most abundant polysaccharide on earth after cellulose. Chitosan gel beads and microspheres can be obtained by cross-linking with polyphosphates and sodium alginate (Anal and Stevens, 2005; Anal *et al.*, 2003). Chitosan coating provides stability to alginate microparticles by providing an additional thickness. This chitosan coated alginate matrix utilizes as an effective encapsulation matrix for therapeutic living cells. The positively charged amino groups of chitosan and negatively charged carboxylic acid groups of alginate, depending on the pH, form a membrane on the microparticle surface, which reduces the leakage of entrapped materials from the particles. The survival and stability of probiotic bacteria loaded into chitosan-coated alginate microparticles are largely dependent on the molecular weight of chitosan. *Lactobacillus bulgaricus* KFRI 673-loaded alginate microparticles were coated with chitosans of three different molecular weights to investigate the survival and stability of *Lactobacillus*

bulgaricus KFRI 673 in simulated gastric fluid (SGF) (pH 2.0) and simulated intestinal fluid (SIF) (pH 7.4). Before encapsulation, the authors examined the survival of free *L. bulgaricus* KFRI 673 in SGF of pH 2.0 and in SIF of pH 7.4. In SGF, none of the cells survived after 60 min (Huguet *et al.*, 1996). On the other hand, survival of the *Lactobacillus* strain was fully maintained in SIF over the time period until 120 min, suggesting that *L. bulgaricus* KFRI 673 is pH sensitive and cannot survive in acidic pH conditions. Therefore, encapsulation of the *Lactobacillus* is essential for its survival when given orally. After encapsulation, the survival of *L. bulgaricus* KFRI 673 was investigated for all microparticle batches after sequential incubation in SGF and SIF. The incubation time in SGF was optimized at 0, 30, 90, and 180 min. After wards, 180 min incubation was carried out in SIF as for sequential incubation. The microparticles prepared with high molecular weight chitosan provided a higher survival rate (46%) compared with the microparticles made with low molecular weight chitosan (36%). Chitosan-uncoated alginate microparticles showed lower survival (25%) of *L. bulgaricus* KFRI 673. The prepared microparticles stability was also investigated at 4°C and 22°C during a four weeks period. Both the free and the encapsulated cells showed similar stabilities at 4°C, whereas high molecular weight chitosan-coated alginate microparticles appreciably improved the *Lactobacillus* stabilities at 22°C compared with free cells and the other respective batches. This was due to the thicker membrane of the microparticles made with high molecular weight chitosan, which protected the encapsulated *Lactobacillus* better than the microparticles made with low and medium molecular weight chitosans and non-encapsulated cells (Lee *et al.*, 2004).

1.4.4 Encapsulation in Cellulose Derivatives

It has been reported that gastric juice resistant tablet formulations of LAB were developed, using hydroxyl propyl methylcellulose acetate succinate (HPMCAS) as well as alginates, apple pectin

and Metolose® as matrix forming components. To optimize the formulation—using survival rate in acid medium and disintegration time in intestinal fluid as test parameters—tablets were modified with respect to LAB content, amount of applied excipients per tablet and compaction forces. A decrease of viable cells of not more than one log unit after 2 h of incubation in acid medium was desired, as well as a disintegration time of 1 h in phosphate buffer pH 6.8. It was found that the amount of HPMCAS in the tablet correlates with gastric juice resistance. As HPMCAS also leads to a decrease of disintegration time in intestinal fluid, slight amounts of this excipient were preferred. The best protective qualities against artificial gastric juice were observed when tablets were prepared from compaction mixtures of LAB, HPMCAS and sodium alginate (Stadler and Viernstein, 2003). In another report they showed the potential use of compression coating as an alternative method for the encapsulation of probiotic bacteria *Lactobacillus acidophilus* to improve their storage stability. Microbial cell containing powders were first compressed into a pellet, which was then encapsulated with a coating material of a combination of sodium alginate and hydroxypropyl cellulose by further compression. The effect of compression pressure on cell viability was studied. Results showed that compression of the microbial cell containing powders at pressures up to 90 MPa caused little loss of viability of the bacteria. Beyond 90 MPa, the cell viability decreased almost linearly with the compression pressure. Further compression to form a coating did not cause significant reduction in the cell viability. The stability of the encapsulated bacteria using the compression pressures up to 60 MPa was approximately 10 times higher than free cell containing powders and cell pellets after 30 days storage at 25°C (Chan and Zhang, 2002). In another report, they used sodium alginate and hydroxypropyl cellulose as a coating material for encapsulation of probiotics in acidic medium. Sodium alginate, which can form gels after being hydrated, has been exploited as the prime coating material. Probiotic cell-containing powders were

first compressed into a pellet, which was then encapsulated with the coating material by further compression. Results indicated significant improvement in the survival of encapsulated cells when exposed to acidic media of pH 1.2 and 2. The encapsulated cells showed 10^4 – 10^5 fold increment in cell survival when compared to free cells under the test conditions. The formation of a hydrogel barrier by the compacted sodium alginate layer has shown to retard the permeation of the acidic fluid into the cells. This contributed to the enhanced cell survival. In addition, it could be deduced from *in vitro* tests that the release of encapsulated cells in the human digestive tract could occur near the end of the ileum and beginning of the colon. The mechanism of cell release is primarily due to the erosion of the alginate gel layer (Chan and Zhang, 2005).

It was reported that cellulose acetate phthalate (CAP) contains ionizable phthalate groups. For this reason, this cellulose derivative polymer is insoluble in acid media at pH 5 and lower but is soluble at pH higher than 6. In addition, CAP is physiologically inert when administered *in vivo*, and is, therefore, widely used as an enteric coating material for the release of core substances for intestinal targeted delivery systems. This was also reported with the encapsulation of *B. pseudolongum* in CAP using an emulsion technique. Encapsulated bacteria survived in higher numbers (10^9 CFU/mL) in an acidic environment than non-encapsulated organisms, which did not retain any viability when exposed to a simulated gastric environment for 1 h. This study was performed using encapsulated *B. lactis* and *L. acidophilus* in CAP polymer by a spray drying method. This work evaluated the resistance of encapsulated microorganisms in acid and high bile salt concentrations. Spray-dried microcapsules of CAP containing *B. lactis* and *L. acidophilus* were effective in protecting both these microorganisms when inoculated into media with pH values similar to those in the human stomach. Encapsulated *L. acidophilus* suffered a reduction of only 1 log at pH 1 after 2 h of incubation, and the population of *B. lactis* was reduced by only 1 log immediately after

inoculation into a pH 1 medium and between 1 and 2 h after inoculation into a pH 2 medium. After inoculation of the CAP microcapsules loaded with bacteria into bile solution (pH 7), complete dissolution of the powder indicated that both the wall material and the process used in the preparation of the microcapsules were adequate in protecting the bacteria, to pass undamaged through the acidic conditions of the stomach, followed by their rapid liberation in the pH of the intestine (Anal and Singh, 2007).

1.4.5 Cellulose Nanocrystals (CNC)

CNC is a cellulosic material, a fascinating biopolymer, subject of intensive research and development and a sustainable raw material for food industry. This nano-material is expected to have a great potential because cellulose materials are the most abundant biological raw materials and can self-assemble into well-defined architectures in multiple scales, from micro to nanosize. Notwithstanding the potential risks of nanoparticles and nanotechnology to human health and the environment, it is important to point out that the CNC is networked in the supra molecular cellulose structure of plant materials. CNC is a very interesting nanomaterial for production of cheap, lightweight, and very strong nanocomposites and this fiber can also be used by the food industry for encapsulation (Klemm *et al.*, 2009). CNC has been shown to be non-toxic by Kovacs *et al.* (2010). This nanomaterial is rod-like in shape and is produced by the controlled hydrolysis of woody plants using sulphuric acid. This treatment results in nano particles which have anionic surface groups on the surfaces, depending on the pH, lead to electrostatic stabilization of the particles in the suspension (Beck-Candanedo *et al.*, 2005).



Figure -1.4: TEM image of highly anionic cellulose nanocrystals adapted from Habibi *et al.*

(2010).

CNC has been investigated as fillers in a number of matrix systems, including siloxanes, poly-(caprolactone), glycerol-plasticized starch, styrene-butyl acrylate latex, cellulose acetate butyrate and epoxies. The motivation for using CNC was sparked by the same interest in other nanocomposite systems utilizing montmorillonite clays and carbon nanotubes, namely: superior properties and very high aspect ratio (Hamad, 2006). CNC offers several potential advantages as a drug delivery excipient. The very large surface area and negative charge of CNC suggests that large amounts of drugs might be bound to the surface of this material with the potential for high payloads and optimal control of dosing. These crystallites bind significant quantities of the water soluble, ionizable drugs such as tetracycline and doxorubicin, which were released rapidly over a 1-day period. Cetyl trimethyl ammonium bromide (CTAB) was bound to the surface of CNC and increased the zeta potential in a concentration-dependent manner from -55 to 0 mV. CNC crystallites with CTAB-modified surfaces bound significant quantities of the hydrophobic anticancer drugs docetaxel, paclitaxel, and etoposide. These drugs were released in a controlled manner over a 2-day period. The CNC-CTAB complexes were found to bind to KU-7 cells, and evidence of cellular uptake was observed (Jackson *et al.*, 2011).

1.4.6 Encapsulation of Probiotics in Whey Protein Isolate (WPI) Gel Particles

Encapsulation of probiotics in whey protein gel particles could offer protection during processing and storage as well as extending the food applications of the living cells and antimicrobials to yoghurt, biscuits, vegetable and frozen cranberry juice. WPI has the potential for the encapsulation of *L. rhamnosus*. Beads were prepared by extruding the denatured WPI-concentrated bacteria solution and 96 % of the probiotic cells were in the whey protein particles. The protein-based technique can provide an alternative for encapsulation with alginate-type gels or spray-coating with fats, the two most widely-used probiotic encapsulation methods. The protein matrix would have different cell release properties than the other encapsulation methods (polymer or fat based). Thus, applications can extend to other foods for protection during processing as well as stability during storage but also in nutraceuticals for protection and cell release in the gastrointestinal tract (Champagne *et al.*, 2006). Shah *et al.* (2012) used whey protein isolate-maltodextrin matrix for encapsulation of an antimicrobial compound (thymol) using the spray drying process.

1.4.7 Encapsulation of Live Probiotics in a Modified Alginate System

Modified alginates were also investigated for encapsulation of live probiotic bacteria to improve their survival in acidic condition. Le-Tien *et al.* (2004) used succinylated alginate and N-palmitoylaminoethyl alginate for probiotic applications. *L. rhamnosus* was microencapsulated into unmodified and modified alginate beads to investigate their acid resistance and viability in acidic condition. To investigate the acid resistance of free cells and encapsulated cells, all the formulations loaded with *L. rhamnosus* were incubated in SGF (pH 1.5) for 30 min. For free cells, the initial count dropped from 1.0×10^8 CFU/ml to an uncountable level after 30 min. Moderate protection was achieved by the unmodified alginate beads loaded with *L. rhamnosus*. Succinylated alginate and succinylated chitosan beads loaded with the probiotic bacteria showed better

protection in SGF, with a slight decrease of viability, although no significant ($P>0.05$) differences were achieved in protection of encapsulated cells between these two formulations. The best protection in SGF was obtained for N-palmitoylaminoethyl alginate with a slight decrease in bacterial cell viability from 2.5×10^7 to 2.2×10^7 CFU/ml. The minor loss of encapsulated cells from N-palmitoylaminoethyl alginate beads could have occurred from near or on the bead surface. N-palmitoylaminoethyl alginate beads showed a promising formulation to protect the live bacteria from an acidic environment and to improve their survival and stability.

1.4.8 Effect of Prebiotics for Probiotic Encapsulate System

Adding the prebiotic inulin to yoghurt boosted the growth of probiotic bacteria and when used in a novel double encapsulation, extended the survival rates of the friendly bacteria. The various prebiotic fibres protect the stability and viability of probiotic *L. rhamnosus* strains during freeze-drying, storage in freeze-dried form and after formulation into apple juice and chocolate-coated breakfast cereals. The studied prebiotics were: sorbitol, mannitol, lactulose, xylitol, inulin, fructooligosaccharide (FOS) and raffinose (Ann *et al.*, 2007).

Incorporation of Hi-Maize starch (a prebiotic) improved encapsulation of viable bacteria as compared to when the bacteria were encapsulated without starch. Inclusion of glycerol (a cryo-protectant) with alginate mix increased the survival of bacteria when frozen at -20°C . The acidification kinetics of encapsulated bacteria showed that the rate of acid produced was lower than that of free cultures. The encapsulated bacteria, however, did not demonstrate a significant increase in survival when subjected to *in vitro* high acid and bile salt conditions. A preliminary study was carried out in order to monitor the effects of encapsulation on the survival of *L. acidophilus* and *Bifidobacterium* spp. in yogurt over a period of 8 weeks. It showed that the survival of encapsulated cultures of *L. acidophilus* and *Bifidobacterium* spp. showed a decline in

viable counts of about 0.5 log over a period of 8 weeks while there was a decline of about 1 log in cultures which were incorporated as free cells in yogurt (Sultana *et al.*, 2000). It was reported that prebiotics (FOS or isomaltooligosaccharides) were used as a growth promoter (peptide) and sodium alginate as a coating material to encapsulate different probiotics such as *L. acidophilus*, *L. casei*, *B. bifidum* and *B. longum*. A mixture containing sodium alginate (1% w/v) mixed with peptide (1% w/w) and FOS (3% w/w) as coating materials produced the highest survival in terms of probiotic counts (Chen *et al.*, 2005).

1.5 Conclusion and Future Challenges

In the food processing industry, encapsulation of probiotics is playing a vital role to protect the viability and enhance the survival of probiotic bacteria against adverse environmental conditions. Encapsulation has also been found to be important for antimicrobial compounds to improve their stability during storage. Encapsulated probiotic bacteria can be used in many fermented dairy products, such as yoghurt, cheese, cultured cream and frozen dairy desserts, and for biomass production. Encapsulation of probiotics and antimicrobials applied in foods on an industrial scale faces technological, microbiological, and financial challenges and also questions linked to consumer acceptance. The main challenge in applying encapsulation of bioactive compounds to new foods to meet consumer interests has to do with finding the appropriate encapsulation technique, safe and effective encapsulating materials. Encapsulation is expected to extend the shelf life of antimicrobials in various food matrices and also increase the heat resistance, compression, shear stress resistance, acid tolerance, stability at room temperature for probiotics. Biopolymers are the best effective materials for encapsulation of bioactives. But when only one biopolymer is used for encapsulation, it does not exhibit an appropriate effect on encapsulation. Mixture of biopolymers could have the best potential for the encapsulation of bioactive compounds.

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1.7 Problematic, Hypothesis and Objectives

The problematic, hypothesis and objectives have been developed on the base of literature review.

The following issues were investigated and developed in this study.

1.7.1 Problematic

- Nisin can readily be degraded in presence of food components or bacterial proteases and essential oils are most volatile components which are very difficult to stabilize in food.
- The uniform application of antimicrobial compounds over the food surface in order to obtain an optimal efficacy of these compounds, while encapsulation could be the effective method to stabilize them.
- Probiotics show a rapid inactivation or degradation during gastric passage and storage especially at room temperature.

Development of a biopolymer systems with an appropriate encapsulation process would overcome these problems.

1.7.2 Hypothesis

- Developed CNC reinforced biopolymer matrix will be utilized for the protection of antimicrobials (nisin and EOs) and probiotics.
- The CNC reinforced biopolymer microbeads will protect nisin and EOs against degradation and volatilization during storage from the contact of food components as CNC has the property to stabilize the EOs in emulsion. The combined microencapsulated nisin and EOs could show a synergy with emerging cold pasteurization techniques like gamma irradiation to protect the safety of RTE foods.

- CNC shows an interesting characteristic with biopolymer microbeads to improve the viability of probiotics during freeze drying. CNC-biopolymer microbeads exhibit the improved viability during storage at room temperature and under gastric treatment. The optimized CNC concentration would be evaluated with other biopolymers for probiotic encapsulation by using central composite design (CCD).

1.7.3 Objectives

- a) To develop and evaluate the CNC reinforcement with alginate matrix by characterizing the physicochemical properties.
- b) To evaluate the antimicrobial properties of the CNC reinforced alginate beads on ready-to-eat (RTE) meat (*in vitro* and *in situ* studies) against *L. monocytogenes*.
- c) To evaluate the possible synergy between active beads and cold pasteurization (Gamma irradiation) to eliminate *Listeria* on RTE meat.
- d) To evaluate the potential reinforcement of alginate beads with CNC to protect the viability of probiotic bacteria (*L. rhamnosus* ATCC 9595) during storage at 4 and 30°C.
- e) Evaluate the effect of CNC with alginate and pectin on the viability of probiotic tablets during storage at 4 and 30°C by using central composite design (CCD).

The methodologies that will be followed for the objectives:

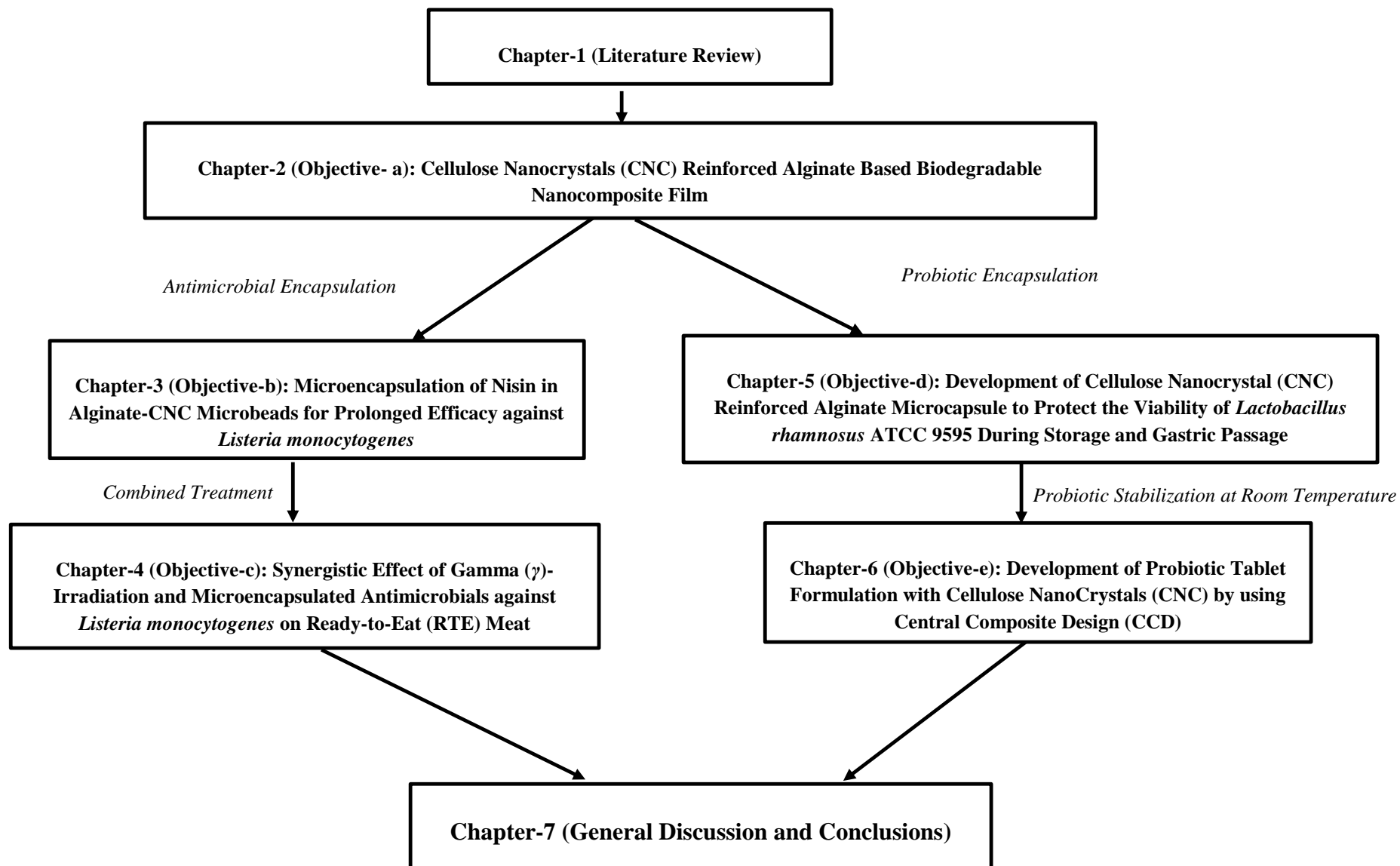
1.7.4 Methodology

- a) For objective a), the physic-chemical properties of films will be carried out by the evaluation of the mechanical characterization (tensile strength, tensile modulus, and elongation break), barrier properties (water vapour permeability, swelling), chemical

characterization (FTIR), thermal analysis (DSC, TGA) and morphological characterization (SEM) will be performed.

- b) For objective b), chemical interaction between alginate-CNC microbeads with nisin will be characterized by FTIR analysis, available nisin concentration (*in vitro*) determined by Bi *et al.* (2011a), antimicrobial activity determined by *in situ* analysis on RTE ham.
- c) For objective c), cold pasteurization or γ -irradiation will be employed with microencapsulated antimicrobials (EOs and nisin) during storage on RTE ham against *L. monocytogenes*.
- d) For objective d), the effect of CNC will be evaluated with alginate microbeads for the protection of *L. rhamnosus* during freeze drying. Two other additives, lecithin and starch, will also be evaluated with alginate microbeads for the protection of *L. rhamnosus* during freeze drying. The mechanical and swelling studies of the optimized microbeads formulations will be checked. The viability of *L. rhamnosus* in microencapsulated optimized microbeads formulations will be evaluated during storage (4 and 30° C) and under gastric treatment.
- e) For objective e), a novel probiotic formulation will be developed by using this CCD. The effect of CNC will be demonstrated in this novel formulation that may show a strong stability of probiotic during storage at room temperature. This novel formulation may also exhibit a controlled release of probiotic through intestine.

1.7.4 Simplified Flowchart for the Ph.D. Thesis



Chapter-2
Publication-1

Cellulose Nanocrystals (CNC) Reinforced Alginate Based Biodegradable Nanocomposite Film

Tanzina Huq¹, Stephane Salmieri¹, Avik Khan¹, Ruhul A. Khan¹, Canh Le Tien¹, Bernard Riedl²,
Carole Frascini³, Jean Bouchard³, Jorge Uribe-Calderon⁴, Musa R. Kamal⁴ and Monique
Lacroix^{1*}

¹Research Laboratories in Sciences Applied to Food, Canadian Irradiation Centre (CIC),
INRS-Institut Armand-Frappier, Université du Québec, 531 Boulevard des Prairies,
Laval, Québec, H7V 1B7, Canada

²Département des sciences du bois et de la forêt, Faculté de foresterie, géographie et géomatique,
Université Laval, Québec, G1V 0A6, Canada

³FPIInnovations, 570 Boulevard St. Jean, Pointe-Claire, Québec, H9R 3J9, Canada

⁴Department of Chemical Engineering, McGill University, 3610 University Street, Montreal, QC
H3A 2B2, Canada

* **Corresponding Author:** Prof. Monique Lacroix

Telephone: +1-450-687-5010; Fax: +1-450-686-5501; **E-mail:** monique.lacroix@iaf.inrs.ca

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2.1 Contribution of the Authors

This article has been published in *Carbohydrate Polymers*. Most of the experimental work, data analysis and draft were prepared by Tanzina Huq with substantial direction from Prof. Monique Lacroix and co-direction from Prof. Bernard Riedl. Prof. Monique Lacroix, my PhD director, checked the protocols during my experiment. Prof. Monique Lacroix and Prof. Bernard Riedl corrected my writing draft. Stephane Salmieri helped to improve the draft of FTIR analysis, thermal analysis and also SEM analysis. Avik Khan helped for XRD draft writing. Dr. Ruhul A. Khan and Dr. Canh Le Tien corrected the main draft. Dr. Carole Frascini and Dr. Jean Bouchard, from FP Innovations, corrected the main draft and gave some suggestions to improve the draft. Dr. Jorge Uribe-Calderon and Prof. Musa R. Kamal performed the thermal and XRD analysis and also helped to analyse the results. Tanzina Huq submitted the articles and both Tanzina Huq and Prof. Monique Lacroix replied to the reviews' comments.

2.2 Specific Objectives of Publication-1

This study was undertaken to evaluate the effect of CNC in alginate matrix for microencapsulation of bioactive compounds. The potential of CNC has been demonstrated for special functional nanomaterials but it is as a biobased reinforcing nanofiller that such nanomaterials have attracted significant interest over the past 20 years. However, the main challenge with nanoparticles is related to their homogeneous dispersion within a polymeric matrix. The specific objectives of this study were to develop the alginate-CNC matrix by characterizing the physico-chemical properties (mechanical, barrier, swelling, thermo-gravimetical and morphological analysis).

2.3 Résumé

Un film nanocomposite à base d'alginate renforcé à l'aide de cellulose nanocristalline (CNC) a été préparé par formation de film par évaporation de solution. La teneur en CNC dans la matrice a été variée de 1-8% (pds / pds % de la matrice). On a trouvé que le nanocomposite renforcé avec 5% en poids du contenu de CNC a présenté la plus haute résistance à la traction, qui a été augmentée de 37% par rapport au témoin. L'incorporation de la CNC a également amélioré de façon significative la perméabilité à la vapeur d'eau (PVE) du nanocomposite montrant une diminution de 31% avec un contenu de 5% en poids de la CNC. Les interactions moléculaires entre l'alginate et la CNC ont été élucidées par spectroscopie infrarouge en transformée de Fourier. Les études de diffraction des rayons X ont également confirmé l'apparition de pics cristallins en raison de la présence de CNC dans les films. La stabilité thermique des films nanocomposites à base d'alginate- a été améliorée après l'incorporation de la CNC.

2.4 Abstract

Cellulose Nanocrystals (CNC) reinforced alginate-based nanocomposite film was prepared by solution casting. The CNC content in the matrix was varied from 1-8 % (w/w % dry matrix). It was found that the nanocomposite reinforced with 5% wt CNC content exhibits the highest tensile strength which was increased by 37% compared to the control. Incorporation of CNC also significantly improved water vapor permeability (WVP) of the nanocomposite showing a 31% decrease due to 5% wt CNC loading. Molecular interactions between alginate and CNC were supported by Fourier Transform Infrared Spectroscopy. The X-ray diffraction studies also confirmed the appearance of crystalline peaks due to the presence of CNC inside the films. Thermal stability of alginate-based nanocomposite films was improved after incorporation of CNC.

Keywords: Alginate, Cellulose Nanocrystals, Nanocomposite, Biopolymers, Morphology, Thermal Properties.

2.5 Introduction

Recent years there have been remarkable developments in the polymeric packaging films for improving the preservation of packaged foods. These films possess the potential for improving stability of foods by acting on the food surface, upon contact (Cha *et al.*, 2002). Biodegradable polymers are known for many decades, these polymers have been ignored mainly because of the low cost of synthetic polymers. However the biodegradable polymers could replace synthetic polymers in many applications, thus reducing the problems of disposability of traditional plastics. In an effort to produce more environment-friendly materials, renewable and biodegradable polymers have been investigated in different fields. Biopolymer films which contain polysaccharide ingredients enable us to obtain edible films with good mechanical and water barrier properties (Briassoulis, 2006).

Alginate is the most widely used material for biopolymeric film. It is a natural polysaccharide derived from marine plants and its basic structure consists of linear unbranched polymers containing β -(1 \rightarrow 4)-linked D-mannuronic acid (M) and α -(1 \rightarrow 4)-linked L-guluronic acid (G) residues (Khan *et al.*, 2010). The chemical composition and sequence of the M and G residues depend on the biological source and the state of maturation of the plant. The stiffness of the three blocks decreases in the order GG > MM > MG. Alginate forms a thermally stable and biocompatible hydrogel in the presence of di- or tri-cations. Alginate is of interest as a potential biopolymer film component because of its unique colloidal properties, which include thickening, stabilizing, suspending, film forming, gel producing, and emulsion stabilizing (Draget *et al.*, 1994; Fabra *et al.*, 2008; Salmieri & Lacroix, 2006; Han *et al.*, 2008).

Cellulose is a fascinating biopolymer, subject of intensive research and development and a sustainable raw material. Cellulose has gained interest as a renewable, environmentally friendly,

and cost-effective reinforcing agent for composite materials (Samir *et al.*, 2005). Cellulose is a natural homopolymer composed of D-glucopyranose units which are linked together by β -(1 \rightarrow 4)-glycosidic bonds (Klemm *et al.*, 2009). Cellulose nanocrystals (CNC) is composed of rod-like shaped nanoparticles, and it can be referred to nanocrystals, whiskers or nanowhiskers. CNC can be prepared from wood pulp by controlled acid hydrolysis. The use of sulphuric acid imparts negative charges to the CNC, thus preventing the CNC particles to aggregate. The CNC suspension is very stable over time. These cellulose nanocrystals are featured by an average diameter of 5–10 nm and an average length of around 100 nm. CNC exhibits very interesting properties such as renewable nature, low density, high specific strength and modulus, large and highly reactive surface which can be used for grafting specific groups. Thus, CNC is very interesting nanomaterial for production of low-cost, lightweight, and very strong nanocomposites (Habibi *et al.*, 2010; Beck-Candanedo *et al.*, 2005).

The main objective of this work was to develop a renewable and biodegradable alginate based nanocomposite films by incorporating CNC for food-packaging applications. The effect of CNC loading on the mechanical, barrier and thermal properties of the alginate matrix was evaluated.

2.6 Materials and Methods

2.6.1 Materials

Sodium alginate (alginic acid sodium salt from brown algae, guluronic acid content ~ 65 - 70%; mannuronic acid content ~5 - 35%) and calcium chloride (granules) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Cellulose Nanocrystals (CNC) was produced in FPIinnovations pilot plant reactor (Pointe-Claire, QC, Canada) from a commercial bleached softwood kraft pulp according to a procedure modified from the literature (Dong *et al.*, 1998).

2.6.2 Preparation of Alginate Based Films

An aqueous solution containing 3 % alginate (w/v) and 1 to 8% (w/w) CNC in dry wt. relative to that of dry matrix was homogenized using a Ultra-Turrax TP18/1059 homogenizer (Janke & Kunkel, Staufen, Germany) at 23°C and 25,000 rpm for 1 min (Salmieri & Lacroix, 2006). Films were cast by pouring 10 mL of the film forming solution into Petri dishes and allowed to dry for 24 h, at room temperature and 35% relative humidity (RH). Then the films were treated with 1% CaCl₂ solution and washed several times with distilled water to remove the excess CaCl₂ present in the films. The films were again dried as described above under the same condition for 6-8 h and the dried water insoluble films were peeled off manually using a spatula and stored in polyethylene bags prior to characterization.

2.6.3 Mechanical Properties Measurement

2.6.3.1 Film Thickness

Thickness of the films (25-35 µm) was measured using a Mitutoyo digimatic Indicator (Type ID-110E; Mitutoyo Manufacturing Co. Ltd, Tokyo, Japan) with a resolution of 1 µm, at five random positions around the film, by slowly reducing the micrometer gap until the first indication of contact.

2.6.3.2 Tensile Strength, Tensile Modulus and Elongation at Break

The films were equilibrated in a desiccator containing a saturated sodium bromide solution ensuring 56% RH at room temperature (21°C) for at least 24 h. Tensile strength (TS), tensile modulus (TM) and elongation at break (Eb%) of the films were measured with a Universal Tensile Machine (Model H5KT, Tinius-Olsen Inc., Horsham, PA, USA,) equipped with a 100 N-load cell (type FBB) and 1.5 kN-specimen grips. The dimensions of the rectangular shape test specimen were 60×15×0.03 mm (L, l, e) with L the length, l the width and e the thickness as recommended

by the standard ISO 14125. Measurements were carried out following an ASTM D638-99 method (1999). Film width was measured using a Traceable[®] Carbon Fiber Digital Caliper (resolution: 0.1 mm/0.01’’; accuracy: ± 0.2 mm/0.01’’; Fisher Scientific, ON, Canada). UTM parameters were set up for "plastics tensile from position" test type with the following selections: 25 mm effective gauge length, flat specimen shape, 1 number of entries, minimum type. The position rate of machine control was fixed to 1 mm/s. Y- and X-axes were assigned to load (100 N-range) and position (500 mm-range) coordinates respectively. TS (maximum stress, MPa) and TM (modulus, MPa) values were automatically collected after film break due to elongation, using Test Navigator[®] program.

2.6.4 Water Vapor Permeability

Water vapor permeability (WVP) tests were conducted gravimetrically using a ASTM 15.09:E96 method (1983). Films were mechanically sealed onto Vapometer cells (No. 68-1, Twing-Albert Instrument Co., West Berlin, NJ, USA) containing 30 g of anhydrous calcium chloride (0% RH). The cells were placed in a Shellab 9010L controlled humidity chamber (Sheldon Manufacturing Inc., Cornelius, OR, USA) maintained at 25° C and 60% RH for 24 h. The amount of water vapor transferred through the film and absorbed by the desiccant was determined from the weight gain of the cell. The assemblies were weighed initially and after 24 h for all samples and up to a maximum of 10% gain. Changes in weight of the cells were recorded to the nearest 10⁻⁴ g. WVP was calculated according to the combined Fick and Henry laws for gas diffusion through coatings and films, according to the following equation:

$$\text{WVP (g.mm/m}^2\text{.day.kPa)} = x\Delta w/A\Delta P$$

where x is the film thickness (mm), Δw is the weight gain of the cell (g) after 24 h, A is the area of exposed film ($31.67 \times 10^{-4} \text{ m}^2$), and ΔP is the differential vapor pressure of water through the film ($\Delta P = 3.282 \text{ kPa}$ at 25° C).

2.6.5 Gel Swelling Property

The test film samples were first dried at 37° C for 12 h in an incubator and then accurately weighed. The dried films (1g) were then immersed in distilled water (10 mL) for 1-8 h. The wet weight of the films was measured by taking out the films from the water and blotting with a filter paper to remove the surface adsorbed water followed by immediately weighing the films. The water uptake or swelling property of the films was calculated by the following equation:

$$S = [(W_s - W_d) / W_d] \times 100$$

Where, S is the percentage of water absorption of the films at equilibrium; W_s and W_d are the weights of the samples in the swollen and dry states, respectively.

2.6.6 Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectra of the films were recorded using a Spectrum One spectrophotometer (Perkin-Elmer, Woodbridge, ON, Canada) equipped with an attenuated total reflectance device for solids analysis and a high linearity lithium tantalate detector. Spectra were analyzed using the Spectrum 6.3.5 software. Films were stored at room temperature for 72 h in a desiccator containing saturated NaBr solution to ensure a stabilized atmosphere of 60% RH at 25° C . Films were then placed onto a zinc selenide crystal, and the analysis was performed within the spectral region of $650\text{-}4000 \text{ cm}^{-1}$ with 64 scans recorded at a 4 cm^{-1} resolution. After attenuation of total reflectance and baseline correction, spectra were normalized with a limit ordinate of 1.5 absorbance units.

2.6.7 X-ray Diffraction

For X-ray diffraction (XRD) analysis, film samples were folded several times to increase the sample thickness. Samples were analyzed between $2\theta = 5^\circ$ and 114° with angle step size $2\theta = 0.02^\circ$ in a D8 Discover X-ray Diffractometer (Bruker AXS Inc., Madison, MI, USA) using a Co K α (40 kV/35 mA) source.

2.6.8 Thermo Gravimetric Analysis (TGA)

Thermo gravimetric analysis of the films was carried out using a TGA 7 (Perkin Elmer, USA) analyzer. Experiments were carried out under nitrogen atmosphere. The weight of the film samples varied from 6-8 mg, scanning range was maintained to $50\text{--}600^\circ\text{C}$ and the heating rate was $10^\circ\text{C}/\text{min}$.

2.6.9 Differential Scanning Calorimetric (DSC) Analysis

Differential scanning calorimetric analysis was carried out using a Pyris DSC calorimeter (Perkin Elmer). The scanning temperature was from 50 to 220°C range. The scanning process comprised an initial heating followed by cooling and finally a second temperature scan was performed. The heating/cooling rate was $10^\circ\text{C}/\text{min}$, under a nitrogen atmosphere.

2.6.10 Scanning Electron Microscopy (SEM) Analysis

Film samples were prepared for scanning electron microscopy by dropping a 5×5 mm piece cut from the center of the film into liquid nitrogen and allowing the piece to equilibrate under the liquid nitrogen. The film piece was fractured into several smaller pieces with a prechilled razor blade held in a vice grip. The samples were deposited on an aluminum holder and sputtered with gold-palladium alloy (gold/palladium deposition rate of 30 s equivalent to coating thickness of approximately 50 \AA) in a Hummer IV sputter coater. SEM photographs were taken with a Hitachi S-4700 FEG-SEM (Hitachi Canada Ltd., Mississauga, ON, Canada) at a magnification of $40000\times$,

at room temperature, equipped with a X-ray detector model 7200 (Oxford Instruments, Abingdon, UK) with a resolution at 1.36 eV to 5.9 keV.

2.6.11 Statistical analysis

To validate the results obtained during different experimental procedure, each analysis was carried out in triplicate in a completely randomized experimental design. An analysis of variance (ANOVA) and multiple comparison tests of Duncan's were used to compare all the results. Differences between means are considered significant when the confidence interval is smaller than 5% ($P \leq 0.05$). The analysis was performed by the PASW Statistics 18 software (IBM Corporation, Somers, NY, USA).

2.7 Results and Discussion

2.7.1 Effect of CNC Loading on the Mechanical Properties of Alginate-Based Films

The tensile strength (TS) of pure alginate films was found to be 57 MPa. **Figure 2.1(a)** shows the effect of CNC content on the TS of alginate-based films. Incorporation of CNC caused a significant ($P \leq 0.05$) increase of TS. With 1% CNC, the TS of the films increased to 65 MPa, an increase of 14% compared to the native alginate film. On the other hand, incorporation of 3, 5 and 8% CNC contents raised the TS of alginate-based films by 25, 37 and 32%, respectively. Here CNC acted as a reinforcing agent in alginate-based films and therefore, it imparts higher TS values to the alginate-based films. The improvement of TS attributed due to good interfacial interaction between CNC and alginate-based matrix because of similar polysaccharide structures of cellulose and alginate. It was also observed that the tensile properties of poly (vinyl alcohol)-based composite films were significantly improved with an increase of CNC loading (Lee *et al.*, 2009). Similarly Chang *et al.* (2010) reported the reinforcing property of CNC with glycerol plasticized starch film.

The tensile modulus (TM) value of the alginate-based films was found to be 1.8 GPa. **Figure 2.1(b)** shows the effect of CNC content on the TM of alginate-based films. Due to the incorporation of 1% CNC into the alginate-based film caused a significant ($P \leq 0.05$) increase of TM to 2.0 GPa, which is an improvement of more than 12% than the native alginate-based films. The highest TM (75% higher than that of the control) was observed for 5% CNC loading. All of the CNC-containing specimens showed higher values of TM than the control sample. CNC reinforced biocomposite films became more brittle as the CNC content increases. The increased TM values of the CNC reinforced alginate-based films may be attributed to the increased stiffness of the films by the addition of CNC. Khan *et al.* (2010) mentioned that the mechanical properties, except elongation, were improved significantly by the addition of cellulose nanocrystals to MC-based films. At low content (5% w/w), CNC could disperse well in the alginate-based matrix, which increases the mechanical properties (TS and TM) of the nanocomposite. However, high content (8% w/w) of CNC may be easily agglomerated, which actually could decrease the effective properties of NCC and facilitate to lower the mechanical properties. **Figure 2.1(c)** shows the effect of CNC content on the elongation at break of alginate-based films. The elongation at break (Eb%) values of the alginate-based films were found to be 10%. A significant decrease down to 5% in the Eb value of the alginate-based films was observed for films having a 5% CNC loading, which represents a relative decrease of 44% compared to that of the control sample. Chang *et al.* (2010) also reported decreased elongations at break with addition of cellulose nanocrystals in plasticized starch-based films.

2.7.2 Water Vapor Permeability

Figure 2.2 shows the effect of CNC concentration on the WVP of the alginate-based films. The values of WVP decreased with the increase in CNC content. The WVP of control alginate-based

films (without CNC) was 5.50 g.mm/m².day.kPa. For all of the alginate-based films loaded with CNC caused lower WVP values compared to those of the control film (i.e. 4.80, 4.44, 3.80 and 3.53 g.mm/m².day.kPa for loadings of 1, 3, 5 and 8% (w/w), respectively). Thus, a significant decrease of more than 31% of WVP was obtained due to only 5% CNC incorporation. Azeredo *et al.* (2009) reported that the addition of at least 10% (w/w) of cellulose nanocrystals within mango puree-based films was effective to decrease WVP significantly. It was also indicated that after incorporation of 10% (w/w) of cellulose nanocrystals, the WVP of the mango puree-based films was improved 24% compared to the control. The presence of CNC is thought to increase the tortuosity in the alginate-based films leading to slower diffusion processes and hence, to a lower permeability. The barrier properties are enhanced if the filler is less permeable than the matrix and has a good dispersion within the matrix. Sanchez-Garcia *et al.* (2010) also demonstrated that after incorporation of 3% (w/w) CNC in carrageenan, the WVP was reduced to 71% compared to the control.

2.7.3 Gel Swelling Property

The gel swelling properties of CNC reinforced alginate-based films presents in **Figure 2.3**. Incorporation of CNC significantly reduced the swelling percentage (S) of alginate-based films. After 1 h, the S value of the control alginate films was found to be 187%, whereas due to the incorporation of 5 and 8% CNC, the S values of the composite films were found to be 99% and 111%, respectively. It was also observed that the S values of the CNC reinforced composite films (5% w/w CNC) decreased about 53% after 8 h of immersion in water than that of the control alginate films. In above experiment, it is reported that WVP of alginate-based films were reduced because of the incorporation of CNC in alginate matrix. So, it was expected to decrease the water uptake of CNC containing alginate films. Ma *et al.* (2009) also reported that the addition of low

content of nanoparticles in glycerol plasticized starch could result in a dispersion that minimized water absorption.

2.7.4 Fourier Transform Infrared Spectroscopy

FTIR analysis of films attempted to characterize the incorporation of CNC into alginate-based film matrix by distinguishing the IR bands and vibrations shifts related to CNC-alginate interactions. For native CNC (**Figure 2.4a**), absorption peaks are mainly assignable to O-H stretching vibration at 3600-3200 cm^{-1} (with typical sharpening at 3335 cm^{-1}), symmetric and asymmetric C-H stretching vibration of aliphatic chains at 2930 cm^{-1} and bound water vibration at 1645 cm^{-1} , as previously described by Khan *et al.* (2010). For native alginate film (**Figure 2.4b**), the absorption bands at 3600-3200 cm^{-1} and 1595 cm^{-1} were assigned to the O-H stretching vibrations and to asymmetric and symmetric COO^- stretching vibration, respectively. In addition, peak at 2930 cm^{-1} were ascribed to overlapping symmetric and asymmetric C-H stretching vibration of aliphatic chains (Han *et al.*, 2008). Some differences can be observed after CNC addition into alginate matrix. Indeed, after addition of 5 and 8% NCC (**Figures 2.4c-2.4d**), a slight increase of typical sharpen peak was observed at 3335 cm^{-1} related to O-H vibration of crystalline CNC. Also, the intensity and width of the overall O-H band (3200- 3600 cm^{-1}) increased with CNC concentration, suggesting an increase of hydrogen bonding between alginate and CNC (Khan *et al.*, 2010). Moreover, typical sharp band at 3335 cm^{-1} appeared and increased with the addition of CNC content.

Other bands at 1160 and 1055 cm^{-1} had their intensity increased after CNC addition. These bands are attributed to typical cellulosic compounds and are assigned to C-O, C-C and ring structures, in addition to external deformational vibrations of C-H, C-OH, C-CO, and C-CH groups, as already mentioned by Ivanova *et al.* (1989). Overall, FTIR spectra of CNC-loaded alginate films provided

qualitative insights into the effect of CNC concentration on the position, width and intensity of IR vibrations related to alginate-CNC interactions. Although many bands from alginate spectra masked typical CNC vibrations, especially in the 1800-1270 cm^{-1} fingerprint region (implying bands related to the degree of order of cellulosic materials). FTIR analysis allowed characterizing CNC-alginate molecular interactions via O-H stretching modes (highly related to the degree of hydrogen bonding) and in the 1160-1050 cm^{-1} region, more associated to deformation vibrations of cellulosic compounds.

2.7.5 X-Ray Diffraction Analysis of the Films

XRD analysis was performed in order to analyze the structural property of the alginate and CNC reinforced alginate films. **Figure 2.5** depicts the diffractograms of CNC, alginate, alginate with 5 and 8% CNC. The diffractogram of alginate consisted of a broad halo (indicator of amorphous nature) containing a sharp peak at around $2\theta = 20^\circ$ indicating crystalline nature. Similar diffractograms for alginate has already reported (Fan *et al.*, 2005; Yang *et al.*, 2000). In counterpart, the diffractogram of CNC showed the characteristic peaks at $2\theta = 16.8^\circ$ and $20-22.4^\circ$ (related to more crystalline order) and corresponding to 110 and 200 planes of cellulose I, respectively, as already reported by Li *et al.* (2009) and Shin *et al.* (2007). Incorporation of 5 and 8% CNC into alginate resulted in the presence of an additional peak at $2\theta = 22^\circ$, relatively to the contribution of CNC that allowed increasing the crystallinity of the films. However, the characteristic peak of alginate at $2\theta = 20^\circ$ disappeared for the nanocomposite with 5% CNC content. This could be due to the formation of a percolated nanocomposite structure through proper dispersion of CNC into alginate matrix. In a percolated nanocomposite a proper dispersion of CNC in the polymer matrix may result in the eventual disappearance of any coherent diffraction peak from the matrix. This phenomenon occurs because the nanocomposite does not present ordering.

The percolated structure facilitates maximum reinforcement due to large surface area in contact with the matrix, as evident from the mechanical strength of the nanocomposite (Favier, & Chanzy, 1995). Also, the intensity of the other peaks at $2\theta=20^\circ$, 38° , 64° and 77° were reduced at 5% CNC content, possibly due to the effect of resonance generated by percolation. Hence, the main contribution of CNC to induce a higher level of crystallinity was observed in the peak at $2\theta=22^\circ$ (proportional increase with CNC content). These results are in agreement with all the results observed previously that described improvement of mechanical, barrier and thermal properties as well as chemical interactions between alginate and CNC determined by FTIR analysis.

2.7.6 Thermal Properties of the Films

Thermo-gravimetric analysis (TGA) and derivative TGA curves for alginate, alginate with 5 and 8% CNC films are represented in **Figure 2.6a** and **2.6b**. The alginate (control) exhibited a decomposition process due to the removal of moisture and surface bound water starting around 206°C (maximum at 222°C) with a maximum thermal decomposition occurring at 257°C . Finally a small change in mass occurred at 388°C with a total remaining mass accounting for 35% (carbonaceous residues). At 5% CNC, the maximum of the initial decomposition occurred at 223°C , followed by the second decomposition (maximum at 261°C). The final carbonaceous content accounted for 36%. At 8% CNC, the maximum of the initial decomposition occurred at 227°C , followed by the second decomposition (maximum at 263°C). The final mass accounted for 35%. Hence, the results showed that TGA curves were shifted towards higher temperatures with CNC content, suggesting better thermal stability of CNC reinforced films which also indicated the strong interactions between CNC and alginate matrix. It was also reported that the thermal stability was improved after incorporation of CNC in chitosan based film and glycerol plasticized starch film (Li *et al.*, 2009 and Chang *et al.*, 2010). Derivative curves (**Figure 2.6b**) are in agreement

with the results observed for TGA curves and clearly indicated the maximum decomposition phase of the films with a slowing in thermal decomposition with CNC content.

The DSC thermograms of alginate, alginate with 5% CNC and alginate with 8% CNC films are presented in **Figure 2.7a** and **2.7b**. The first DSC heating cycle shows that all films evolved in similar trends but the heat flow was increased with CNC content (especially at a higher content of CNC). Indeed, a decomposition process was observed from around 210°C (for control) to 218°C (for 8% CNC reinforced alginate film). Similar decomposition was observed by TGA analysis. The second DSC heating cycle (**Fig 2.7b**) indicates that the curves did not reveal either crystalline structure or other transition phase. These observations do not point out any thermal decomposition. This is most probably due to the degradation of components during the first heating. Therefore, TGA and DSC curves showed that the addition of CNC in alginate films contributed to a substantial improvement in their thermal stability up to 227° C.

2.7.7 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was carried out for extensive morphological inspection of cross-section in CNC reinforced film. **Figure 2.8** represents the SEM images of alginate film (**a**), alginate film with 5 % w/w CNC (**b**) and alginate film with 8 % w/w CNC (**c**), at 40,000× magnification of the fractured surface (cross-section) of the film. From the SEM image of alginate film (**Figure 2.8a**), it was found that the structure of alginate was composed of interlayer organization, suggesting filamentous (fibrillous) aspect and no susceptible filling as compared to samples containing CNC (5% CNC and 8% CNC). The structure of 5 % CNC reinforced alginate film (**Figure 2.8b**) was overall similar to control but interlayers were arranged more closely, with a finer filamentous aspect. Moreover the filling structure looked more compact in presence of CNC. The improved mechanical properties of alginate film with 5% CNC may be attributed to this

compact unidirectional interlayer arrangement. Similarly, Azeredo *et al.* (2009) also reported that the incorporation of CNC in mango puree film showed a compact unidirectional arrangement. For alginate film with 8 % CNC (**Figure 2.8c**), the structure was more disorganized (heterogeneous) with the presence of fracture, up and down (serration) shape and less lamellar organization as compared to 5% CNC. These observations could be due to the presence of more CNC aggregates inserted into alginate network and suggesting that CNC has kept much of its original physical properties. Indeed, Chang *et al.* (2010) reported that higher loading of cellulose nanocrystals did not disperse well in plasticized starch-based films. Overall these SEM observations seem to support all structural modifications and improvements of mechanical and thermal properties related to CNC incorporation in alginate films. Analogous SEM images of CNC into polysaccharide matrices were reported by other studies.

2.8 Conclusion

It appears that CNC was well dispersed in alginate matrix. So, incorporation of small amount of CNC (5% w/w) significantly increased the mechanical and barrier properties of the alginate-based matrix. After incorporation of 5% CNC in alginate matrix, tensile and barrier properties were improved compared to the control alginate film. FTIR analysis characterized a molecular interaction between alginate and CNC due to the hydrogen bonding and thermal properties were also improved after incorporation of CNC. Overall, it was optimized that small amount (3-5% w/w) of CNC had a significant impact on the improvement of physicochemical and thermal properties of alginate-based films.

2.9 Acknowledgement

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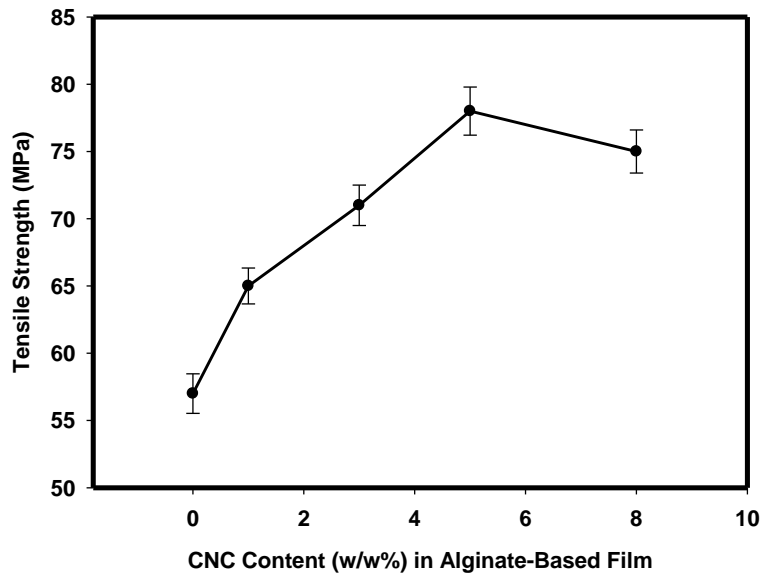


Figure 2.1(a): Effect of CNC Content (w/w %) on Tensile Strength (MPa) of Alginate-based Film, as a function of CNC content in dry matrix.

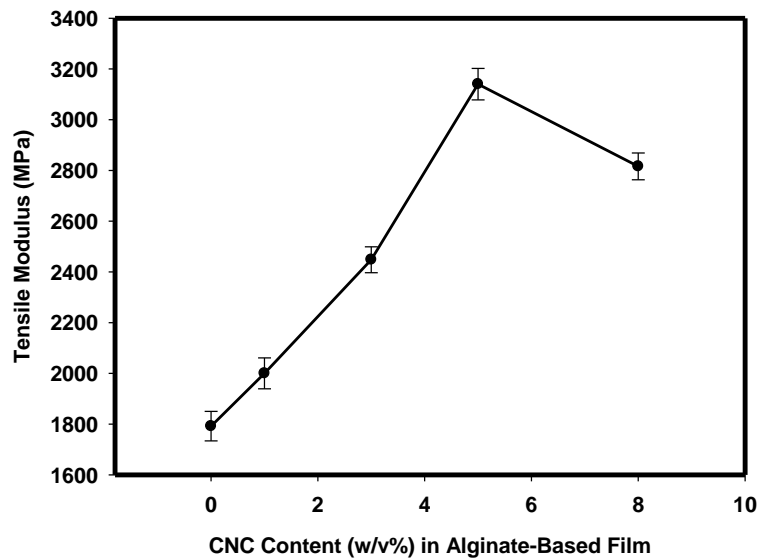


Figure 2.1 (b): Effect of CNC Content (w/w %) on Tensile Modulus (GPa) of Alginate-based Film, as a function of CNC content in dry matrix.

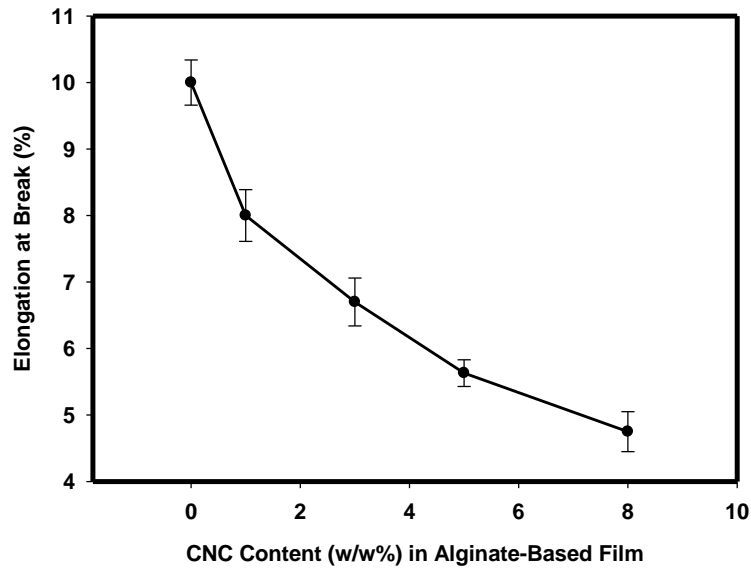


Figure 2.1(c): Effect of CNC Content (w/w %) on Elongation at Break (%) of Alginate-based Film, as a function of CNC content in dry matrix.

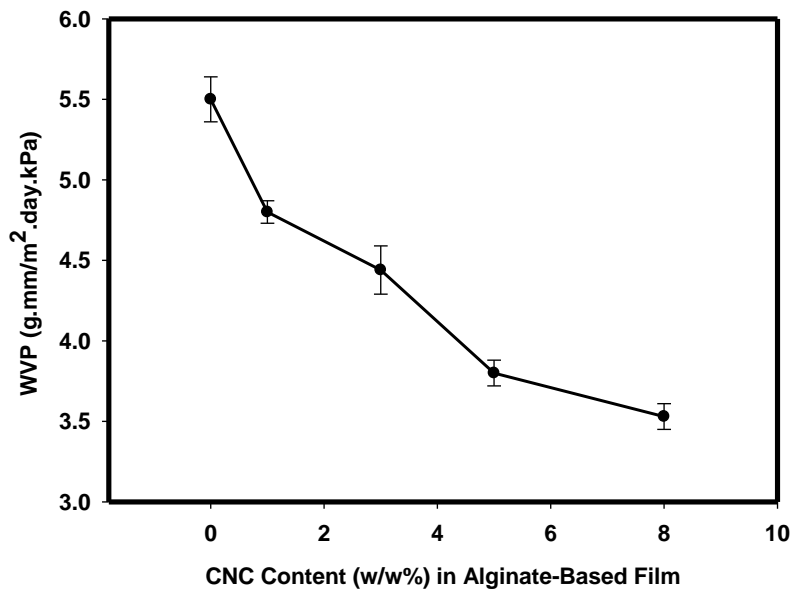


Figure 2.2: Effect of CNC Content (w/w %) on WVP of Alginate-based Film, as a function of CNC content in dry matrix.

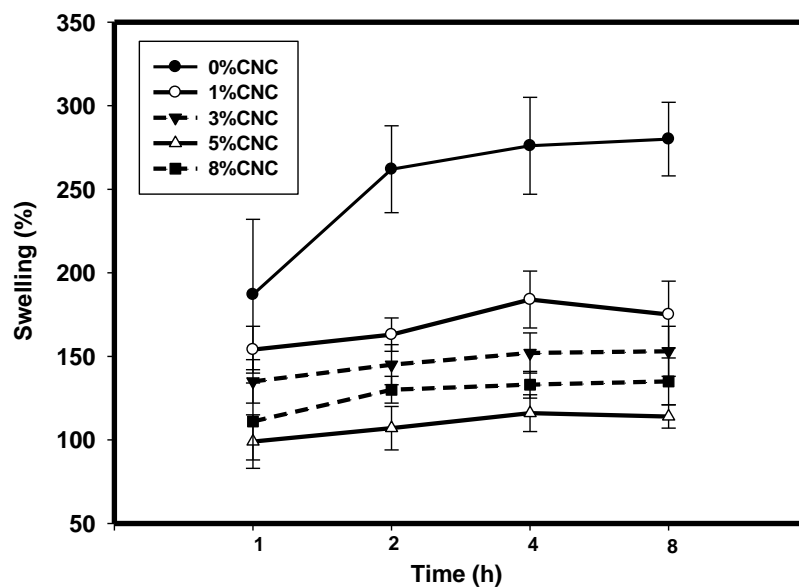


Figure 2.3: Effect of CNC Content (w/w %) on Swelling of Alginate-based Film, as a function of CNC content in dry matrix.

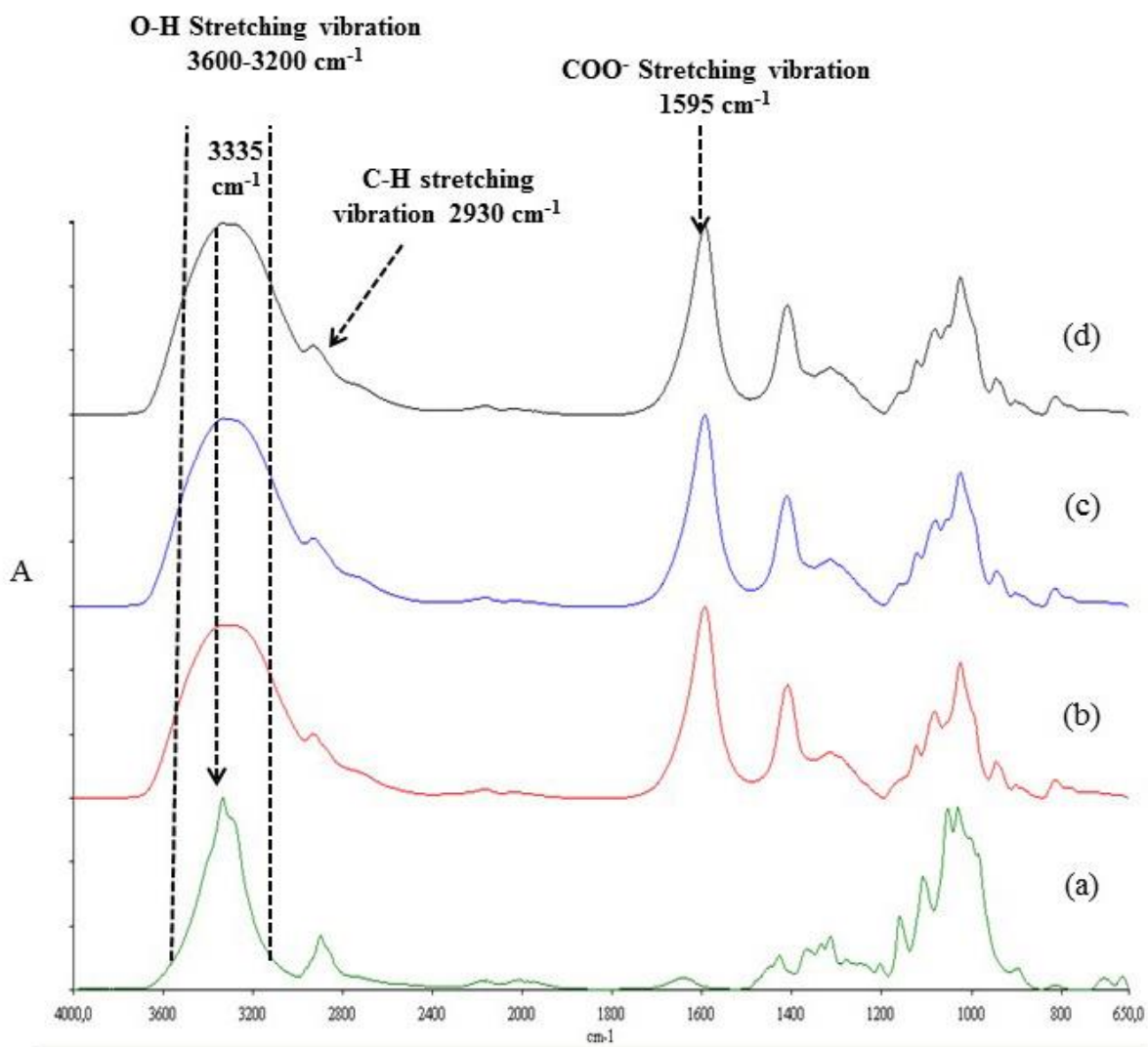


Figure 2.4: FTIR spectra of (a) Pure CNC film, (b) Native alginate, (c) Alg+5% (w/w) CNC and (d) Alg+8% (w/w) CNC.

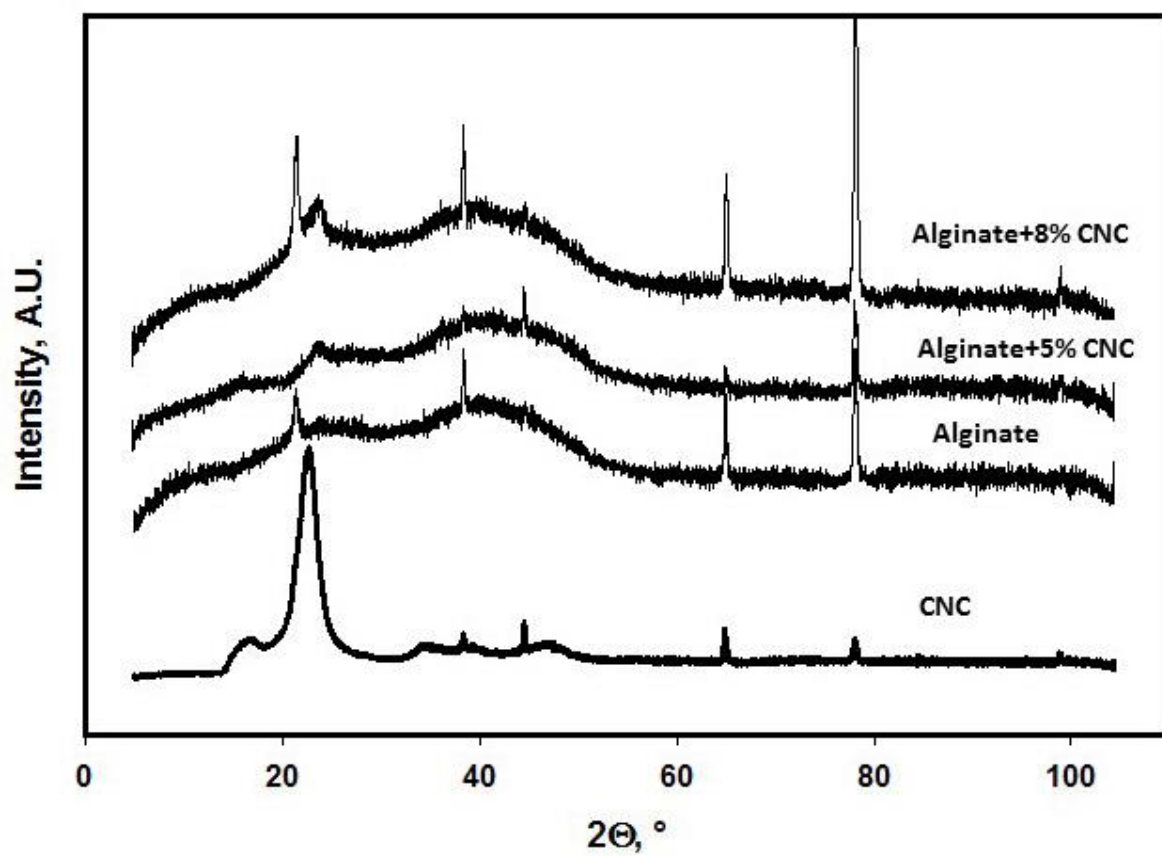


Figure 2.5: X-ray Diffractograms for CNC, Alginate, Alg+5% (w/w) CNC and Alg+8% (w/w) CNC.

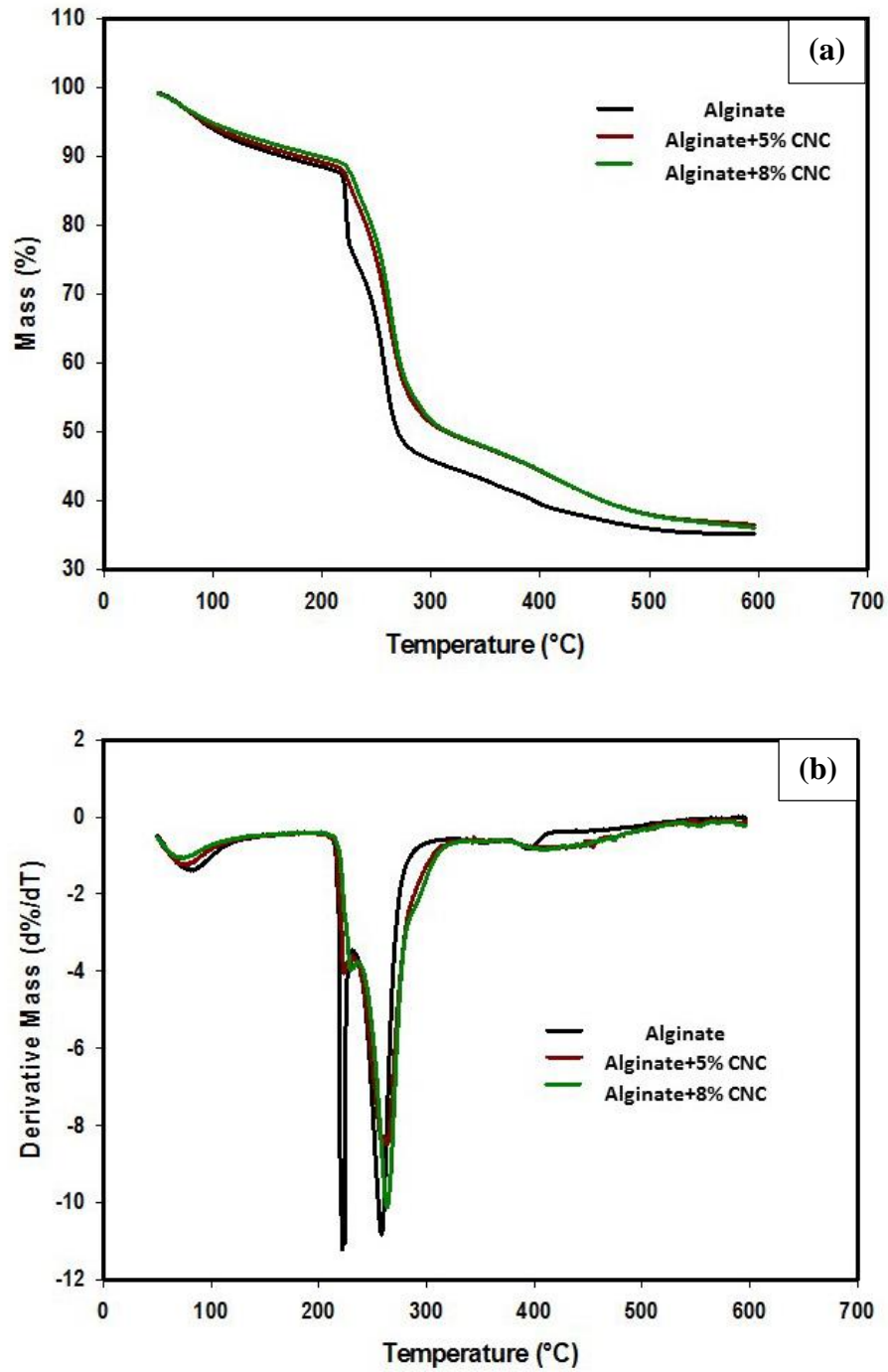


Figure 2.6: (a) TGA and (b) derivative TGA curve for Alginate, Alginate +5% (w/w) CNC and Alginate +8% (w/w) CNC Film.

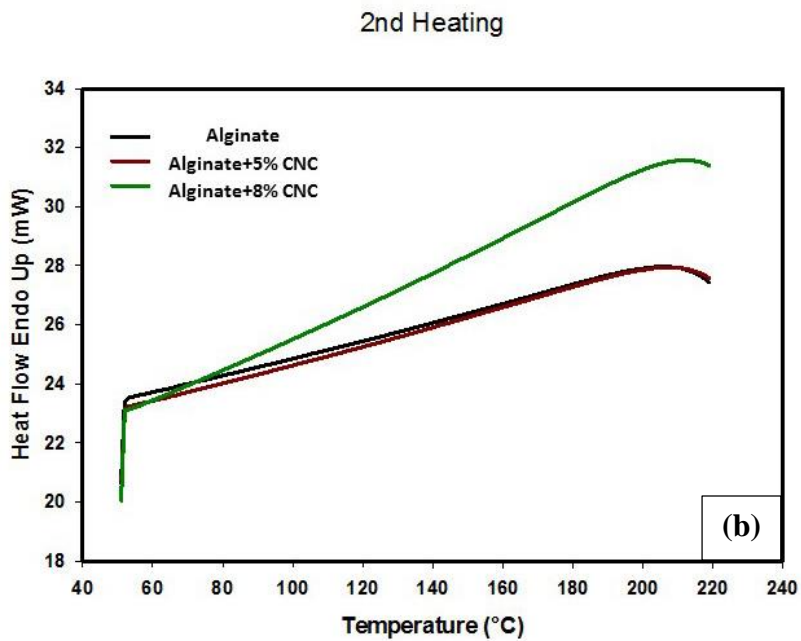
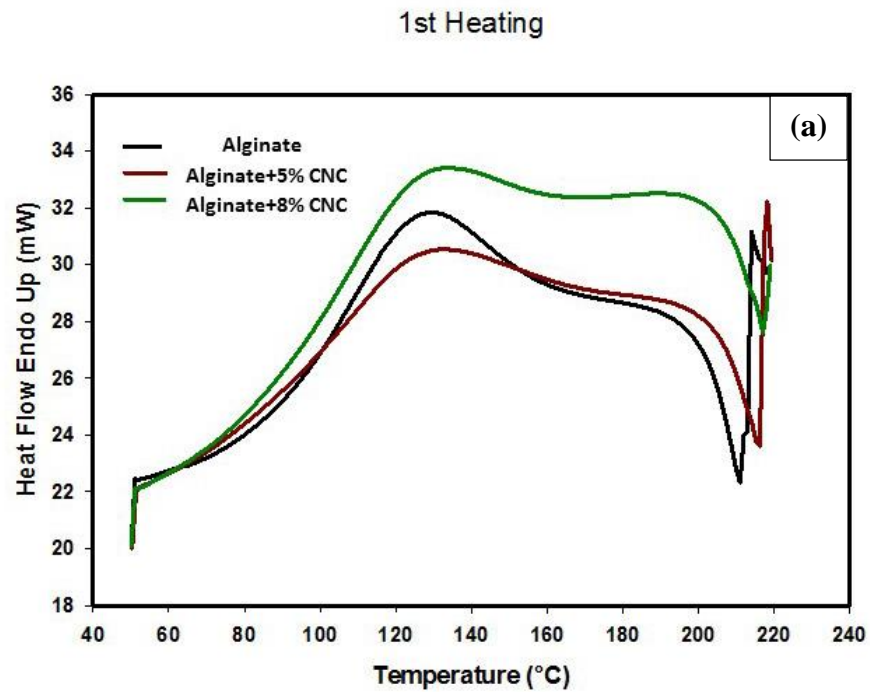


Figure 2.7: DSC curves for alginate, alginate with 5 and 8% (w/w) CNC films (a) first heating and (b) second heating.

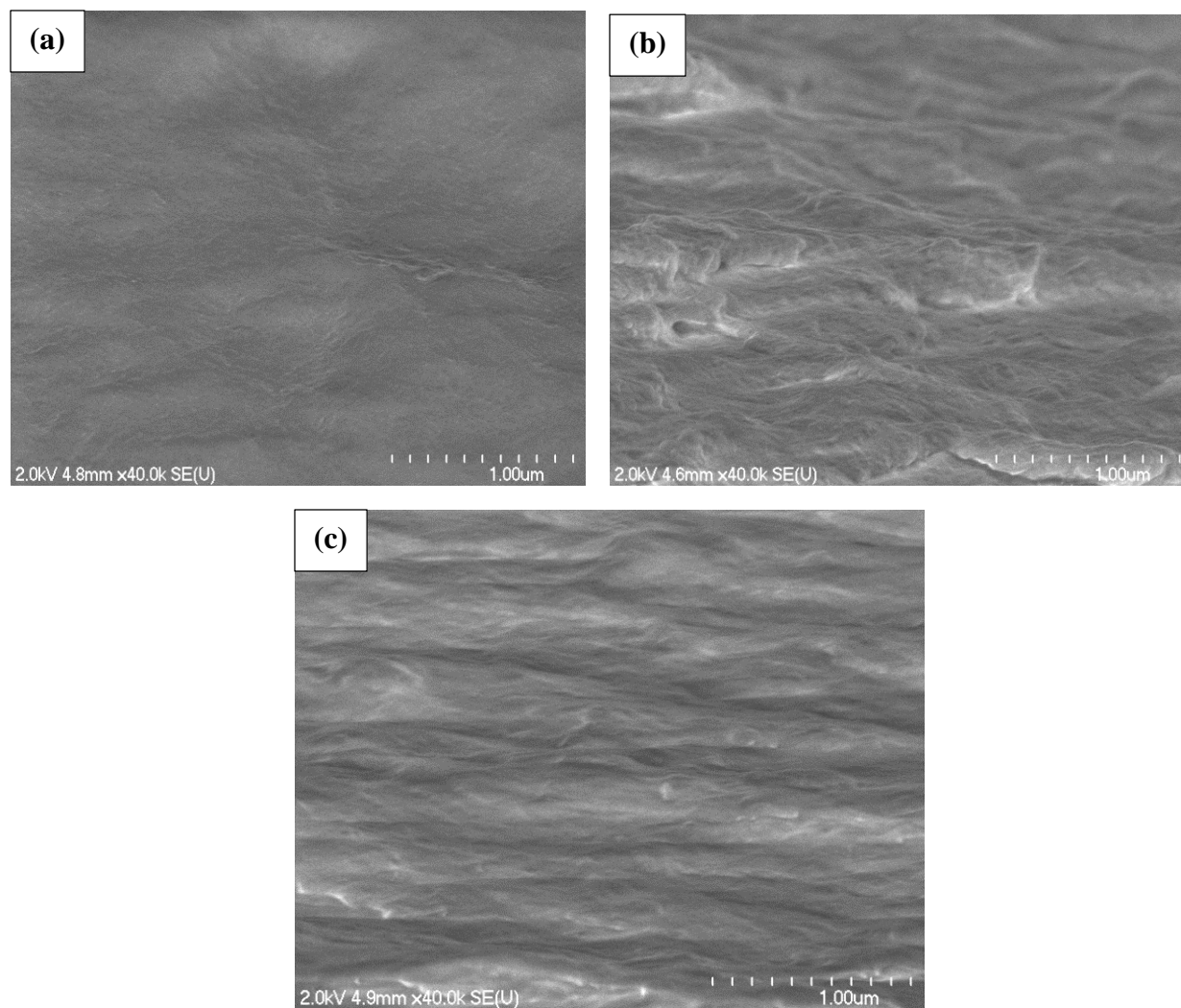


Figure 2.8: SEM images of the fracture surface of alginate film (a), alginate film with 5 % (w/w) CNC (b) and alginate film with 8 % (w/w) CNC (c).

2.11 General Discussions of Publication-1

The effect of CNC in alginate matrix was optimized in this study. Results demonstrated that 5 % (w/w) in alginate matrix improved 37 and 75% of TS and TM, respectively. This results revealed that incorporation of 5 % (w/w) in alginate matrix enhanced the mechanical properties of the alginate matrix. Incorporation of 5 % (w/w) in alginate matrix improved the barrier properties of the matrix. However, 5 % (w/w) in alginate also showed a change in thermo-gravimetical and morphological analysis. This optimized alginate-CNC matrix was further used for microencapsulation of bioactive compounds in food applications.

Chapter-3
Publication 2

Microencapsulation of Nisin in Alginate-Cellulose Nanocrystal (CNC) Microbeads against *Listeria monocytogenes*

Tanzina Huq¹, Bernard Riedl², Jean Bouchard³, Stephane Salmieri¹ and Monique Lacroix^{1*}

¹Research Laboratories in Sciences Applied to Food, Canadian Irradiation Centre (CIC),
INRS-Institut Armand-Frappier, Université du Québec, 531 Boulevard des Prairies,
Laval, Québec, H7V 1B7, Canada

²Département des sciences du bois et de la forêt, Faculté de foresterie, géographie et géomatique,
Université Laval, Québec, G1V 0A6, Canada

³FPIInnovations, 570 Boulevard St-Jean, Pointe-Claire, Québec, H9R 3J9, Canada

* **Corresponding Author:** Prof. Monique Lacroix

Telephone: +1-450-687-5010; Fax: +1-450-686-5501; **E-mail:** monique.lacroix@iaf.inrs.ca

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3.1 Contributions of the Authors

All the experiments, data analysis and draft have been accomplished by Tanzina Huq with the continuous direction of Prof. Monique Lacroix, my PhD director. During my experiments, all the protocols were checked by Prof. Lacroix. Both Prof. Lacroix and Prof. Bernard Riedl (co-director) have helped me to improve the discussions during writing and they have also corrected the main draft of the publication. Dr. Jean Bouchard has helped me to check and correct the draft. Stephane Salmieri has helped me to perform the FTIR experiment.

3.2 Specific Objectives of the Publication-2

Microencapsulation of bioactive agents such as nisin is developing its interest in biotechnology based food application. Nisin is an antimicrobial polypeptide containing 34 amino acids and has shown a strong antimicrobial effect against *Listeria monocytogenes*. It has been found that direct addition of nisin in ready-to-eat (RTE) meat showed less antimicrobial activity during storage. Microencapsulation of nisin has been found an intelligent technology to improve the antimicrobial activity during storage. Biopolymers are widely used as an encapsulating matrix. Our optimized alginate-CNC matrix was utilized as an encapsulating matrix in Publication-2. The objectives of this publication were to develop a new microencapsulation technology for nisin and evaluate the antimicrobial activity of microencapsulated nisin by *in vitro* and *in situ* studies.

3.3 Résumé

La présente étude a été menée afin de développer des perles comestibles comportant de la nisine micro-encapsulée afin d'inhiber la croissance de *L. monocytogenes* dans le jambon prêt-à-manger (RTE). Des concentrations différentes de la nisine (16, 31 et 63 µg/ml) ont été micro-encapsulées dans un système de perles polymère alginate-CNC (Cellulose Nanocristalline). La microencapsulation a gardé la nisine 20 fois plus disponible (63 µg/ml) que la nisine (63 µg/ml) libre pendant 28 jours de stockage à 4 ° C. Les résultats ont montré que la nisine microcapsulée (63 µg/ml) demeure disponible avec un contenu en nisine de 31.26 µg / ml disponible après 28 jours de stockage à 4 ° C alors qu'il n'y avait pas de contenu disponible dans le cas de la nisine libre, après le même délai. Les tranches de jambon cuit ont ensuite été enrobés par les perles de nisine micro-encapsulés, inoculées avec *L. monocytogenes* (~ 3 log UFC/g) et stockées à 4 ° C dans des emballages sous vide pendant 28 jours. Les billes ou perles contenant 16, 31 et 63 µg/ml de nisine t ($P \leq 0.05$) ont réduit significativement la croissance de *L. monocytogenes* de 2.65, 1.50 et 3.04 log UFC/g, respectivement, après 28 jours de stockage par rapport à la nisine libre. En outre, la micro-encapsulation de nisine dans les perles n'a pas modifié les propriétés physico-chimiques (pH et couleur) des jambons RTE pendant le stockage.

3.4 Abstract

The present study was undertaken to develop nisin-microencapsulated edible beads in order to inhibit the growth of *L. monocytogenes* in ready-to-eat (RTE) ham. Different concentrations of nisin (16, 31 and 63 µg/ml) were microencapsulated into alginate–CNC (Cellulose Nanocrystal) beads. Microencapsulation increased the available nisin (63 µg/ml) concentration by 20 times compared to free nisin (63 µg/ml) during 28 days of storage at 4° C. Results showed that microencapsulated nisin (63 µg/ml) exhibited 31.26 µg/ml available nisin content after 28 days of storage at 4° C whereas there was no available nisin content left for free nisin. The cooked ham slices were then coated by the microencapsulated nisin beads, inoculated with *L. monocytogenes* (~3 log CFU/g) and stored at 4° C under vacuum packaging for 28 days. The beads containing 16, 31 and 63 µg/ml of nisin significantly ($P \leq 0.05$) reduced the growth of *L. monocytogenes* by 2.65, 1.50 and 3.04 log CFU/g after 28 days of storage compared to free nisin. Furthermore, microencapsulated nisin beads did not change the physicochemical properties (pH and colour) of RTE ham during storage.

Key Words: Microencapsulation, Nisin, Microbead gel coating, *Listeria monocytogenes*, RTE meat, Cellulose Nanocrystal

3.5 Introduction

The safety of ready-to-eat (RTE) meat products is of high concern due to the likelihood of contamination by dangerous food-borne pathogens such as *L. monocytogenes*. This contamination mainly occurs during the post processing stage. The RTE meat products such as cooked ham are completely processed prior to final packaging and are consumed without further cooking which makes them susceptible to contamination by pathogenic bacteria. *L. monocytogenes*, a gram positive, non-spore rod shaped bacteria, is responsible for the food borne disease called listeriosis (Mbandi and Shelef, 2002). Scallan *et al.* (2011) reported that *L. monocytogenes* caused 94% hospitalization resulting in a 15.6% death rate in the US each year. In 2008, there was a listeriosis outbreak in Canada and 22 people died due to the contamination of Maple Leaf Food products (meat products) by *L. monocytogenes* (CNN, 2008). The symptoms of listeriosis are fairly variable and range from a mild-flu like illness to more serious complications such as meningitis, septicemia, stillbirths and abortions. In general, listeriosis is restricted to pregnant women, infants, the elderly and people with weak immune systems but has been reported to have a high mortality rate (Juck *et al.*, 2010). Based on the characteristics of the *L. monocytogenes* and related diseases, FDA (US Food and Drug Administration) maintains a “zero-tolerance” policy for *L. monocytogenes* in RTE meat products (Anonymous, 1994; Shank *et al.*, 1996). The RTE meats contain some salts such as sodium chloride, nitrite and nitrate that have antimicrobial activities but they do not inhibit the growth of *L. monocytogenes* during storage at refrigerated temperatures (Coma, 2008). Thus *L. monocytogenes* is halotolerant and can tolerate a high level of salt up to 16%. It is also psychrotrophic bacteria and can grow in temperatures as low as 1°C. It can survive in a wide range of pH (4.2-9.6) and is a facultative anaerobe (Lungu and Johnson, 2005; Farber and Peterkin, 1991). Due to this nature, it is very difficult to control the growth of *L. monocytogenes* during

storage and distribution of RTE meat products. Therefore, it is necessary to take additional measures such as addition of antimicrobial compounds to control the adverse effect of this pathogen in RTE meat.

Various methods have been proposed to control the post processing contamination on RTE meat products and it is found that antimicrobial coating is one of the most innovative techniques to control the growth of *L. monocytogenes* during storage condition on RTE meat. Nisin is an antimicrobial polypeptide or bacteriocin of 34 amino acids, produced by several strains of *Lactococcus lactis* and recognized as GRAS (generally recognized as safe) by the United States Food and Drug Administration (FDA) as stated in the Code of Federal Regulations (CFR section 184.1538) (Millette *et al.*, 2007). It is a cationic, hydrophobic and amphiphilic peptide, with antibacterial activity against many Gram-positive bacteria such as *L. monocytogenes*. Nisin has been used throughout the world to preserve the meat products (Chen and Hoover, 2003; Nguyen *et al.*, 2008). Direct addition of nisin to food surfaces may lead to some loss of its activity, because it migrates towards the center of the food which can result in an associated dilution and depletion of its effect (Nguyen *et al.*, 2008; Quintavalla and Vicini, 2002). Also direct addition of nisin may be inactivated by proteolytic enzymes of meat components such as Glutathione S-transferase (GSTs) (Rose *et al.*, 1999). Stergiou *et al.* (2006) reported that the inactivation rate (%) of nisin was higher in fresh meat than in cooked meat. They have also demonstrated that nisin showed more than a 50% of inactivation after 4 days of storage in cooked meat products. Hence, microencapsulation with food biopolymers would be the best technology to keep its antimicrobial activity during storage. Alginate is one of the most widely used biopolymers for microencapsulation of bioactive compounds (nisin) due to its biocompatibility, GRAS status and non-toxicity. Sodium alginate is the salt of alginic acid, a linear (1–4) linked polyuronic acid

extracted from brown seaweed (Pawar and Edgar, 2012). Alginate can easily form microbeads by cross-linking with di- or tri-cations such as CaCl₂. The material microencapsulated on the inert alginate environment could be delivered at a desired rate in a controlled release system. Microencapsulated compounds are released from the alginate matrix by diffusional processes that are facilitated by the action of the polymeric network (Goh *et al.*, 2012). In our current study, the alginate-CNC matrix that was reported in Chapter-2 (Huq *et al.*, 2012) was used for the microencapsulation of nisin. Cellulose nanocrystals (CNC) are a cellulose based nanomaterial which is composed of rod-like shaped nanoparticles. CNC is a new nanomaterial in food industry and it has been found to be non-toxic (Alexandrescu *et al.*, 2013). Thus, microencapsulation of nisin in alginate-CNC microbeads would be an innovative antimicrobial vehicle for RTE meat products against *L. monocytogenes*.

The aim of this study was to evaluate the potential of alginate-CNC microbead containing nisin to inhibit the growth of *L. monocytogenes* during different time intervals. Firstly, the chemical characterization of alginate-CNC microbead containing nisin and nisin alone were evaluated by Fourier Transform Infrared (FTIR) Spectroscopy analysis. Then, the antimicrobial activity of free and microencapsulated nisin against *L. monocytogenes* was evaluated by *in vitro* model (Nisin Bioassay) using different concentrations of nisin (16, 31 and 63 µg/ml). The antimicrobial efficiency of the free nisin and microbeads containing nisin was also evaluated *in situ* using ham as a RTE meat model during storage at 4° C. Thirdly, the physicochemical characteristics (pH and color) of the ham samples coated with microbeads was evaluated during 28 days of storage at 4° C.

3.6 Materials and Methods

3.6.1 Materials

Sodium alginate (alginic acid sodium salt from brown algae, guluronic acid or glucuronic content ~ 65 - 70%; mannuronic acid content ~5 - 35%), calcium chloride (granules) and lactic acid were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). CNC was supplied from FPInnovations which was produced in their pilot plant (Pointe-Claire, QC, Canada) from a commercial bleached softwood kraft pulp according to a procedure modified from the literature (Dong *et al.*, 1998). Nisin (Niprosin™, purity 2.5%, 77.5% salt and 20% vegetable protein, Profood, Naperville, IL, USA) was purchased from Pro-food International Inc.. Brain heart infusion (BHI) broth and Palcam agar were purchased from Alpha Biosciences Inc. (Baltimore, MD, USA). Preservative salts such as sodium chloride, triphosphate, erythorbate and nitrite salt were delivered from BSA Food Ingredients (St-Leonard, Quebec, Canada). Ground lean pork meat was purchased from a local grocery store (IGA, Laval, Quebec, Canada).

3.6.2 Preparation of Nisin Solution

Different concentrations of nisin (0.25, 0.5 and 1% w/v) were prepared by dispersing Niprosin™ powder in 100mL 0.01M CaCl₂ solution and the pH of the nisin-CaCl₂ solution was adjusted to 3-3.5 by diluted lactic acid. Nisin-CaCl₂ solution was stirred overnight for proper mixing. After stirring overnight, the nisin-CaCl₂ solution was centrifuged for 15 min at 3500×g at 4°C to remove the undissolved particles and the nisin-CaCl₂ supernatant was collected.

3.6.3 Microencapsulation of Nisin

An aqueous suspension was prepared by dissolving 3% (w/v) of alginate in deionized water for 24hr under magnetic stirring. A 1% (w/v) CNC suspension was prepared by dispersing spray dried CNC powder in deionized water under magnetic stirring. Then, the CNC suspension was subjected to ultra-sonication (QSonica Q-500, Misonix, Qsonica, LLC, Newtown, CT, USA) at 1000 J/g of CNC. A 5 % (w/w) CNC from 1 % CNC suspension (according to wt% of alginate) was homogenized using an Ultra-Turrax TP18/1059 homogenizer (Janke and Kunkel, Staufen, Germany) at 23°C and 25000 rpm for 1 min. The different concentrations of nisin-CaCl₂ solutions (16, 31 and 63 µg/ml) were mixed with alginate-CNC suspension (alginate-CNC:nisin-CaCl₂ : 75:25) to form the antimicrobial gel microbeads according to Rajaonarivony *et al.*, (1993). The activity of free nisin during time was also verified in order to compare with the microencapsulated nisin. Free nisin solution was prepared in distilled water without CaCl₂ following the same process. The formulations are presented as follows, N1-16 µg/ml (free nisin), N2-31 µg/ml (free nisin), N3-63 µg/ml (free nisin) and N1-E 16 µg/ml: microencapsulated 16 µg/ml nisin, N2-E 31 µg/ml: microencapsulated 31 µg/ml nisin, N3-E 63 µg/ml: microencapsulated 63 µg/ml nisin.

3.6.4 Fourier Transform Infrared (FTIR) Spectroscopy Analysis for Alginate-CaCl₂-Nisin

Complex

FT-IR spectra of microencapsulated nisin in alginate microbeads were recorded using a Spectrum One spectrophotometer (Perkin-Elmer, Woodbridge, ON, Canada) equipped with an attenuated total reflectance device for solids analysis and a high linearity lithium tantalate detector. Spectra were analyzed using the Spectrum 6.3.5 software. Microbeads were dried onto a zinc selenide crystal for 15-20 min and the analysis was performed within the spectral region of 650-4000 cm⁻¹

with 64 scans recorded at a 4 cm⁻¹ resolution. After attenuation of total reflectance and baseline correction, spectra were normalized with a limit ordinate of 1.5 absorbance units.

3.6.5 Bacterial Culture

Five strains of *L. monocytogenes* HPB (Health Canada, Health Products and Food Branch (HPB), Ottawa, Canada) were used in this experiment. All bacterial strains were isolated from contaminated food samples: HPB 2558 1/2b from beef hot dogs, HPB 2818 1/2a from homemade salami, HPB 1043 1/2a from raw turkey, 2569 1/2a from cooked cured sliced turkey and HPB 2371 1/b from raw turkey. The microorganisms were kept frozen at -80°C in BHI broth containing glycerol (10% v/v). Before use, the stock cultures were propagated twice through 2 consecutive 24 h growth periods in BHI broth at 37°C to obtain the working cultures containing approximately 10⁹ CFU/mL.

3.6.6 BHI-Agar Deep-Well Model to Evaluate Depletion Activity of Nisin (in vitro Study)

The BHI-agar deep well model was adapted from Bi *et al.* (2011a). To prepare the BHI-agar deep-well model for nisin depletion test, a solution containing BHI solids (3.7%) and agar (1.0%) was autoclaved for 20 min at 121°C. The solution (225mL) was then poured into a 600-mL beaker to a height of 40mm. After gel solidification, four wells (from gel surface to bottom) were made in each beaker using a 7.0- mm pipet tip. Subsequently, 1.0 mL of each concentration of free and microencapsulated nisin preparation was added to each well. After immediate addition of free and microencapsulated nisin at day 0 and after 1, 7, 14, 21 and 28 days of storage at 4°C, a 100-μL aliquot of each nisin preparation was transferred from the well to a bioassay plate to determine the available nisin content. **Figure 3.1** represented the BHI-Agar Deep well method.

3.6.7 Nisin Bioassay against *L. monocytogenes*

Microencapsulated and free nisin activity, either for those freshly prepared or stored in BHI-agar deep-well, was determined as described by Pongtharangkul and Demirci (2004) with some modifications. The BHI-agar with Tween 20 (1.0%) was prepared and autoclaved. After cooling, the solutions were inoculated with 1.0% (v/v) BHI broth containing *L. monocytogenes* (10^6 CFU/mL). A 25 mL of inoculated broth was added to each petri dish plate (95mm×15mm) and allowed to solidify. Thereafter, holes 7.0mm in diameter were made and 100- μ L of microencapsulated or non-microencapsulated nisin preparation was added to each agar well. The plates were incubated for 24 h at 37°C temperature and the area of the inhibitory ring was measured to determine the activity against *L. monocytogenes*.

To evaluate the available nisin (0-63 μ g/ml), standard curve was conducted by measuring the area of inhibitory rings of nisin depletion (Bi *et al.*, 2011b). During storage, the available nisin (μ g/ml) was calculated from the regression equation of the standard curve.

3.6.8 Preparation of Ham Samples (in situ Study)

Ham was prepared following a model developed in our laboratories (Dussault *et al.*, 2014). Ground lean pork was cooked in presence of different preservatives such as sodium chloride (1.5%), tripolyphosphate (0.43%), sodium erythorbate (750 ppm) and sodium nitrite (50 ppm) salts for 1hr at 162.7° C in a cooking oven. After cooking, the ham was removed from the oven and placed at 4° C for 24 hr. Then, the cooked ham was sliced and 8 ml of free or microencapsulated nisin was spread over each ham slice. The coated cooked ham slice was then inoculated with ~3 log CFU/g *L. monocytogenes* and vacuum packaged within 24hr of production. The samples were stored at 4° C up to 28 days. Ham samples were periodically taken for microbial analysis during the storage time.

3.6.9 Microbiological Analysis

Each ham sample was homogenized for 1 min in sterile peptone water (0.1% w/v) in a lab-blender 400 stomacher (Seward Medical, London, UK). From this homogenate, appropriate serial dilutions were prepared in 0.1% peptone and 100 µl of each dilution was spread on a palcam plate. Palcam agar supplemented with acriflavine (5 mg/mL), polymyxin B (10 mg/mL) and ceftazidime (8 mg/mL) for the selective enumeration of *L. monocytogenes*. The plates were incubated at 37 °C for 48 h. Following incubation, the colony-forming-units (CFU) were counted using a magnifier. Detection limit for all microbial analysis was 50 CFU/g of meat. All bacterial counts were transformed to logarithmic (log₁₀) values.

3.6.10 pH Measurement of Ham Samples

The pH value of ham coated with microencapsulated or free nisin was determined during storage at 1, 14 and 28 days (Gitrakou *et al.*, 2010). Cooked ham samples (10 g) were blended with 90 mL of distilled water for 1 min using a blender (IKA-Laboratory T-25, Germany) and the pH values were determined with a pH meter (Accumet Basic AB15, Fisher Scientific, ON, Canada). The pH meter was calibrated with standard buffer solutions (pH 4 and 7).

3.6.11 Colour Measurement of the Ham Samples

Color measurements were performed on ham surface coated with microencapsulated and free nisin using a Colormet (Instrumar Ltd, Newfoundland, Canada) using the CIELAB system. Measurements were taken three times on each sample and the mean values were used to determine the color coordinates L^* , a^* , b^* where L^* axis represents the lightness from black ($L^* = 0$) to absolute white ($L^* = 100$), a^* axis varies from green (-) to red (+) and b^* axis from blue (-) to yellow (+) (Giroux *et al.*, 2001).

3.6.12 Statistical Analysis

To validate the results obtained during different experimental procedure, each analysis was carried out in triplicate in a completely randomized experimental design. An analysis of variance (ANOVA) and multiple comparison tests of Duncan's were used to compare all the results. Differences between means were considered significant when the confidence interval was smaller than 5% ($P \leq 0.05$). The analysis was performed by the PASW Statistics 19 software (IBM Corporation, Somers, NY, USA).

3.7 Results and Discussions

3.7.1 ATR-FTIR Analysis of Alginate-CNC Microbead Containing Nisin

ATR-FTIR has been used for the structural analysis of microencapsulated nisin. Figure-3.2.A shows the FTIR spectra of Ca-alginate-CNC microbeads containing 16, 31 or 63 $\mu\text{g/ml}$ of nisin. For alginate-CNC microbeads without nisin (**Figure 3.2A-i**), the characteristic absorption bands were observed at $3600\text{-}3200\text{ cm}^{-1}$ (due to O-H stretching vibration), 2934 cm^{-1} (C-H stretching vibration), 1599 cm^{-1} (asymmetric and symmetric COO^- stretching vibration) and 1401 cm^{-1} (symmetric COO^- stretching vibration). Previous study ascribed that a slight increase of typical sharpen peak was observed at 3335 cm^{-1} related to O-H vibration of crystalline CNC which suggesting an increase of hydrogen bonding between alginate and CNC (Huq *et al.*, 2012).

Nisin incorporated microbeads showed a narrowing of band at O-H stretching vibration. Typical characteristic peaks for nisin were found around 1599 cm^{-1} due to CO-NH bending vibration (peptide bonds) and 1401 cm^{-1} due C-H bending vibration (hydrocarbon chains). Thus, the peak around 1599 cm^{-1} and 1401 cm^{-1} of alginate-CNC microbeads with nisin became sharper and shifted to higher wave number (**Figure 3.2A-ii, iii and iv**). In addition, the absorption area between the regions $1718\text{-}1497\text{ cm}^{-1}$ also reduced (from 100 to 89) by addition of nisin (from 16 to 63

µg/ml) (**Figure 3.2B**). The decrease in area could be attributed to the electrostatic interaction between positively charged nisin and negatively charged COO⁻ of alginate. The pKa of alginic acid (alginate) is 3.5 and isoelectric point of nisin is 8.8, thus during microbead preparation at neutral pH; alginate and nisin show an electrostatic interaction (Hosseini *et al.*, 2014). These results are also in good agreement with Zohri *et al.* (2010) where the authors demonstrated nisin interaction with alginate-chitosan microparticles. Similar interaction between nisin-stearic acid (Sebti *et al.*, 2002) and nisin- hydroxypropyl methylcellulose (Sebti *et al.*, 2003) was also reported.

3.7.2 Microencapsulated and Free Nisin Availability during Storage: in vitro Study

In the current study, the release activity of free and microencapsulated nisin was evaluated by BHI-agar deep-well model (*in vitro*) during storage at 4 °C and the diffusion test was done by checking the inhibitory ring against *L. monocytogenes*. BHI is a nutritious medium that supplies proteins and other nutrients to support the growth of microorganisms and can be considered as a simulated *in vitro* food model. BHI contains infusions from calf brain and beef heart, proteose and peptone, dextrose, sodium chloride, and disodium phosphate. Bi *et al.* (2011a) and Bi *et al.* (2011b) have reported that BHI-containing broths and gels lead to a rapid depletion of peptide activity. In the BHI-agar deep-well method, the prepared hole was filled with free and microencapsulated nisin with different concentrations (16, 31 and 63 µg/ml). The antimicrobial peptide molecules diffuse from the free and microencapsulated antimicrobial nisin solution into the gel (causing diffusion-based depletion) and BHI components diffuse from the agar gel into the solution (causing irreversible antimicrobial active compounds adsorption or degradation).

The availability of free and microencapsulated nisin was evaluated (*in vitro*) by the evaluation of *L. monocytogenes* growth inhibition using the BHI-agar deep-well model during storage at 4 °C. A 2-order polynomial “standard curve” for free (N) and microencapsulated (N-E) nisin was used

(**Figure 3.3 A and B**) to correlate the area of the inhibitory ring with the amount of available nisin. **Figure 3.4 A) and B)** present the results of the available nisin content from free (N) and microencapsulated (N-E) nisin during storage at 4 °C. **Figure 3.5 A and B** represents the digital photograph of agar diffusion assay against *L. monocytogenes* for free and microencapsulated nisin during storage. After 1 day of storage, the available nisin of N1-16µg/ml, N2-31µg/ml and N3-63µg/ml (free nisin) was 2.1, 6 and 11 µg/ml, respectively. After 14 days of storage, free nisin formulation N1-16µg/ml and N2-31µg/ml drastically lost their antimicrobial activity and the available nisin was found to be 0.12 µg/ml for both N1-16µg/ml and N2-31µg/ml. Formulation N2-63µg/ml showed only 2.61µg/ml of available nisin after 14 days of storage and lost its antimicrobial activity after 28 days of storage.

After 1 day of storage, microencapsulated nisin formulations show an available nisin of 38, 63 and 89 µg/ml for N1-E16µg/ml, N2-E31µg/ml and N3-E63µg/ml, respectively. After 14 days of storage, the available nisin on N1-E16µg/ml, N2-E31µg/ml and N3-E63µg/ml was respectively 21, 37 and 61 µg/ml. Thus, microencapsulation of 63µg/ml of nisin (N3-E63µg/ml) exhibited 20 times more available nisin as compared to free nisin (N3-63µg/ml). After 28 days of storage, all microencapsulated nisin formulations still showed available nisin. A value of 18.38, 20.59 and 31.26 µg/ml of available nisin was found for N1-E16µg/ml, N2-E31µg/ml and N3-E63µg/ml, respectively, after 28 days of storage. Our study increased the available nisin for microencapsulated formulations during storage compared to both Bi *et al.* (2011a) and Bi *et al.* (2011b) studies due to the activity of novel carrier (alginate-CNC microbead) of nisin. Bi *et al.* (2011b) found the available nisin (initial concentration 150 µg/ml) in carbohydrate nanoparticles 19.4 µg/ml after 20 days of storage. However, our formulation with initial concentration 63 µg/ml (N3-E63µg/ml) showed a strong protection during 28 days of storage and the available nisin

content was 31.26 µg/ml. Wan *et al.* (1997) reported that nisin in alginate microparticles showed a good activity compared to free nisin during storage but they used this formulation for the reduction of *Lactobacillus curvatus* in skim milk. This technique is interesting due to convenient application on the target system which can be manipulated for desirable loading and retention of antimicrobial peptide. The same concept had already been used for drug (Doxorubicin) delivery (Rajaonarivony *et al.*, 1993).

3.7.3 Antimicrobial Activity of Microencapsulated Nisin against *L. monocytogenes*: *in situ*

Test

The antimicrobial effect of free and microencapsulated nisin against *L. monocytogenes* was evaluated on cooked ham as a RTE meat model (**Figure 3.6 A and B**). The effectiveness of nisin to prevent the growth of *L. monocytogenes* was dependent on the nisin concentration. After 4 weeks (28 days) of storage at 4° C, there was no significant ($P > 0.05$) difference in the bacterial growth between the control ham (8.24 log CFU/g) and control ham coated microbeads without nisin (8.25 log CFU/g) samples. The microencapsulated nisin showed better antimicrobial effect as compared to free nisin during storage. The ham slices coated with N1-16µg/ml, N2-31µg/ml and N3-63µg/ml free nisin exhibited a respective bacterial count of 7.18, 5.24 and 4.73 log CFU/g, after 4 weeks of storage at 4° C. Similarly, ham slices coated with N1-E16µg/ml, N2-E31µg/ml and N3-E63µg/ml microencapsulated nisin showed respective bacterial counts of 4.53, 3.74 and 1.69 log CFU/g, after 4 weeks of storage at 4° C. Thus, the microencapsulation of nisin significantly ($P \leq 0.05$) reduced *L. monocytogenes* growth by 2.65, 1.50 and 3.04 log CFU/g in presence of 16, 31 and 63µg/ml of nisin respectively, as compared to the free nisin after 4 weeks of storage at 4° C. The level of *L. monocytogenes* was also lower than the detection limit (≤ 50 CFU/g) for the microencapsulated beads containing 63µg/ml of nisin (N3-E63µg/ml) during the whole storage period. The

microencapsulation of nisin also increased the lag phase of bacterial growth during storage. Lag phase is defined as a microbial kinetics which is typically observed by a delayed response of the microbial population to a (sudden) change in the environment (Swinnen *et al.*, 2004). In the current study, formulation N2-31 µg/ml and N3-63 µg/ml (free nisin) presented a 7 day lag phase of bacterial growth and after 7 days, the bacteria started to grow exponentially during storage. Whereas, microencapsulated formulation N2-E3 1 µg/ml showed a 14 days lag phase of bacterial growth during storage and during 28 days of storage, no bacterial growth was detected using formulation N3-E63 µg/ml.

The external surface of cooked ham slice acts as a primary site of contamination by *L. monocytogenes* during processing (slicing and packaging) and the equipment, personnel, and other surfaces with which the product comes in contact, become contaminated and, in turn, these serve as secondary sources of contamination. *L. monocytogenes* contamination of preserved products such as cooked ham renders it a high-risk product as pronounced uninhibited growth of the organism is a potential risk. *L. monocytogenes* is a psychrotrophic pathogen that is able to grow on cooked ham at a refrigeration temperature of 4°C (Stekelenburg and Kant-Muermans, 2001). It is surmised that antimicrobials such as nisin when directly applied on the surface may diffuse much faster throughout the product lowering the local surface concentration to sub-active levels. On the contrary, edible antimicrobial coatings maintain the necessary preservative concentration at the product surface for a relatively longer period of time (Coma, 2008). Nisin is a ribosomally synthesized and post-translationally modified antimicrobial lantibiotic. It contains 34 amino acids distributed in clusters of bulky hydrophobic residues at the N-terminus and hydrophilic residues at the C-terminus end (Asaduzzaman and Sonomoto, 2009; Van De Ven *et al.*, 1991). As nisin was microencapsulated in alginate matrix, the hydrophilic C-terminal of nisin could be engaged by the interaction with

hydrophilic alginate matrix which was already demonstrated by FTIR analysis. Also the N-terminus of nisin contains two positive charges on that domain (Lins *et al.*, 1999). Thus, this positively charged hydrophobic N-terminus of nisin can electrostatically interact with negatively charged phosphate groups on target cell wall precursor lipid II by forming pores (Cleveland *et al.*, 2001). Though there is a debate over the types of pore formation by nisin, with most groups favoring the “barrel-stave” (Ojcius and Young, 1991) or “wedge” models (Driessen *et al.*, 1995). But the general steps involving for nisin antimicrobial activities include i) binding to the bacterial membrane, followed by ii) insertion of the peptide into the bacterial membrane, iii) pore formation and iv) interactions of nisin with lipid II (Karam *et al.*, 2013). Millette *et al.* (2007) revealed that encapsulated nisin in activated calcium alginate (500 IU/g) beads showed 1.77 log reduction against *Staphylococcus aureus* on ground beef during 14 days of storage but for free nisin the authors showed 2.21 log CFU/g reduction. Juck *et al.* (2010) reported that an alginate coating with nisin (500 IU/g) and sodium diacetate (0.25%) on RTE turkey meat products reduced 1.10 log CFU/g the growth of *L. monocytogenes* during 21 days of storage at 4°C. Nguyen *et al.* (2008) showed ~2 log CFU/g counts of *L. monocytogenes* after 14 days of storage on frankfurters for nisin (25000IU/mL) encapsulated in a cellulosed based matrix. Comparing these results, the present study revealed that alginate microbeads with nisin (N3-E63µg/ml) could completely inhibit the bacterial growth of *L. monocytogenes* compared to control ham coated microbeads where a 6.55 log reduction was observed after 28 days of storage.

3.7.4 Effect of Microencapsulated Nisin on Ham pH During Storage

RTE cooked ham samples without alginate microbeads (control ham) showed pH values of 6.3, 6.4 and 6.3 after 1, 14 and 28 storage days, respectively (**Figure 3.7 A**). However, cooked ham samples coated with microbeads without nisin showed 5.6, 6.1 and 6.2 pH values at day 1, 14 and 28 storage

respectively (**Figure 3.7 B**). It was found that at day 1, the alginate microbead coating significantly ($P \leq 0.05$) reduced the pH value of ham samples as compared to control ham without microbeads and without nisin. After 14 and 28 days of storage, the pH value of ham coated with alginate microbeads was not significantly affected ($P > 0.05$). Chidanandaiah et al., 2009 reported that an alginate coating on buffalo meat patties did not change pH values during storage which represented a similar trend with our study. RTE cooked meat samples coated with free nisin (different concentration) showed pH values around 6.2-6.5 up to 28 days of storage (**Figure 3.7 A**) that was not significantly ($P > 0.05$) different from the control ham. Similarly, microencapsulated nisin (different concentration) in alginate microbead coated RTE cooked ham samples also showed the pH values around 6.2-6.4 (**Figure 3.7 B**) during whole storage period (28 days). Though at day 1, alginate microbeads coating without nisin reduced ($P \leq 0.05$) the pH value (5.6) but microencapsulated nisin coated microbeads showed the pH value 6.3 which was similar to the control ham pH value. This study revealed that free and microencapsulated nisin has no effect on pH value of cooked ham. Authors also reported that bacteriocin-coated plastic packaging film did not affect the pH values of ground beef during storage (Kim *et al.*, 2002).

3.7.5 Colour of RTE Ham during Storage

The effect of free and microencapsulated nisin on colour coordinates L^* , a^* and b^* of RTE cooked ham was evaluated during storage and the values are presented in **Table 3.1 A and B**. In control ham samples (without nisin and without microbeads), there was no significant ($P > 0.05$) change on L^* - value during storage and the mean L^* - value was 68. Ham samples coated with microbeads without nisin showed a higher L^* -value after 28 days of storage ($P \leq 0.05$) showing a mean value of 70.2. The L^* -value of ham coated with free nisin was not significantly ($P > 0.05$) different from the control ham after 28 days of storage. A mean L^* -value of 66.2, 66.5 and 65.6 was observed for N1-

16µg/ml, N2-31µg/ml and N3-63µg/ml, respectively. There was also no change on L^* -value between samples coated with microencapsulated nisin during storage. After 28 days of storage, a^* -value of control ham coated microbead without nisin was increased ($P \leq 0.05$) compared to day 1. The mean value of 10.11 and 12.5 was found for day 1 and 28 days of storage, respectively. The a^* -value did not show any change during storage for free and microencapsulated nisin coated RTE ham samples. Both free and microencapsulated nisin bead coated RTE ham samples did not change their b^* -value during storage.

Color is a significant indicator of the RTE meat quality, because it is one of the most important features influencing evaluation of meat by the consumer. Whereas, myoglobin is the principal protein responsible for meat color, hemoglobin and cytochrome C can also play a role on meat color. Myoglobin is a water soluble protein containing eight α -helices (A–H) linked by short non-helical sections (León *et al.*, 2006; Chmiel *et al.*, 2011; Mancini and Hunt, 2005a). During food processing or storage, air can bind with this myoglobin molecule on the exposed meat surfaces to form oxymyoglobin by oxidation. This pigment gives meat its bright red color (Kamarei *et al.*, 1979; Giroux *et al.*, 2001). Mancini *et al.* (2005b) reported that cooked pork developed a red or dark pink colour during storage. Our present findings revealed that nisin coatings (both free and microencapsulated) could reduce this oxidation of stored cooked ham due to the vacuum packaging (96% vacuum and 4% air) of RTE meat samples that was followed our whole experiment. Indeed, nitrite salts (50 ppm used our current study) are often added to meat especially cured meats as it forms an irreversible red complex with hemoglobin or myoglobin, thus hiding any browning or oxidation effects and erroneously giving the impression to the customer the meat is fresh (Sindelar and Milkowski, 2011).

3.8 Conclusion

The major finding of this work is that microencapsulation of nisin in alginate-CNC microbead can be used as an edible coating for RTE cooked ham to inhibit the growth of *L. monocytogenes* during storage. Microencapsulation of nisin (63µg/ml) increased the lag phase of bacterial growth up to 28 days. The molecular characterization revealed the interaction between alginate-CNC and nisin which also demonstrated the better retention activity of microencapsulated nisin during storage. Our findings further established the importance of microencapsulation of antimicrobial agents compared to the conventional direct addition method.

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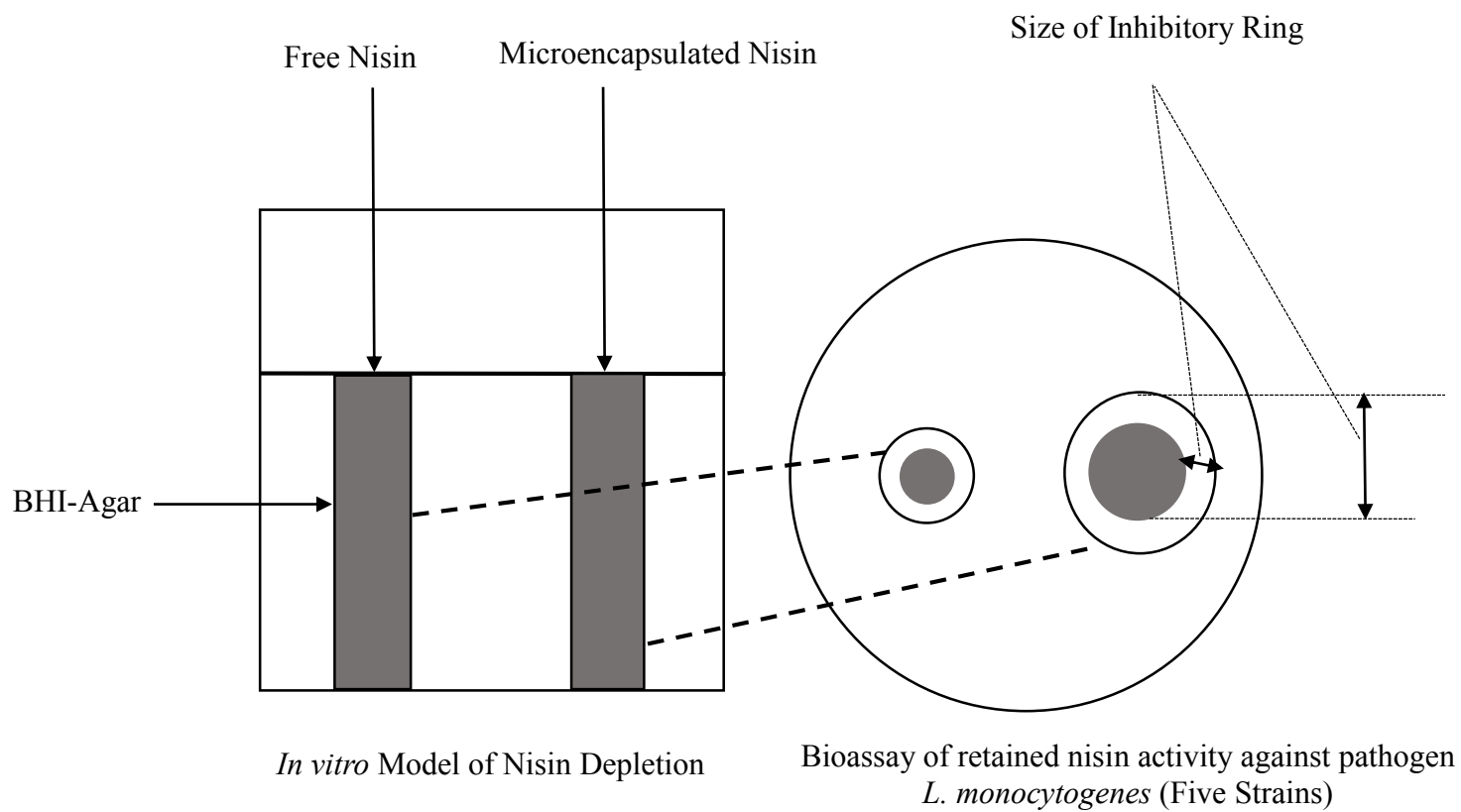


Figure 3.1: BHI-agar deep-well model of peptide depletion during storage (left) and activity bioassay against a pathogen (right) which is adapted from *Bi et al.* (2011a).

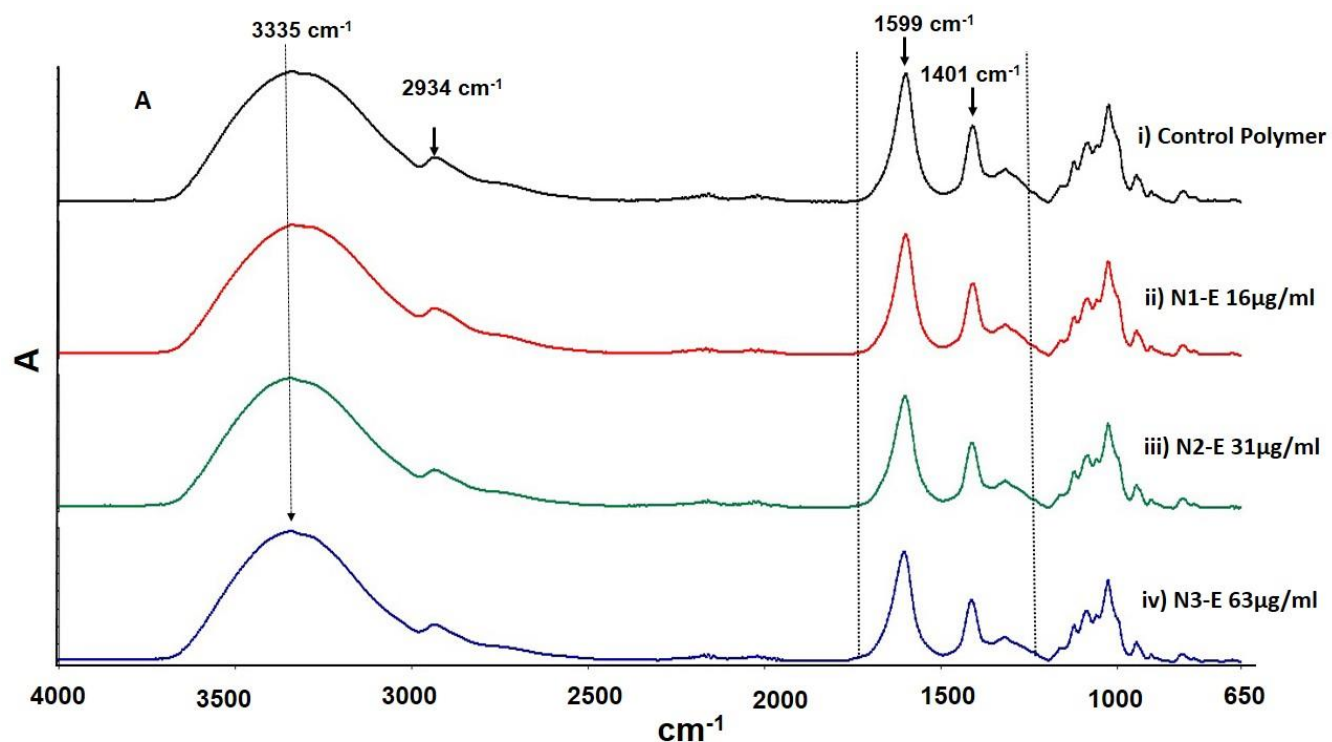


Figure 3.2 A: ATR-FTIR spectra of i) control polymer (alginate-CNC microbead); ii) N1-E 16 $\mu\text{g/ml}$ (alginate-CNC microbead with 16 $\mu\text{g/ml}$ of nisin); iii) N2-E 31 $\mu\text{g/ml}$ (alginate-CNC microbead with 31 $\mu\text{g/ml}$ of nisin); iv) N3-E 63 $\mu\text{g/ml}$ (alginate-CNC microbead with 63 $\mu\text{g/ml}$ of nisin).

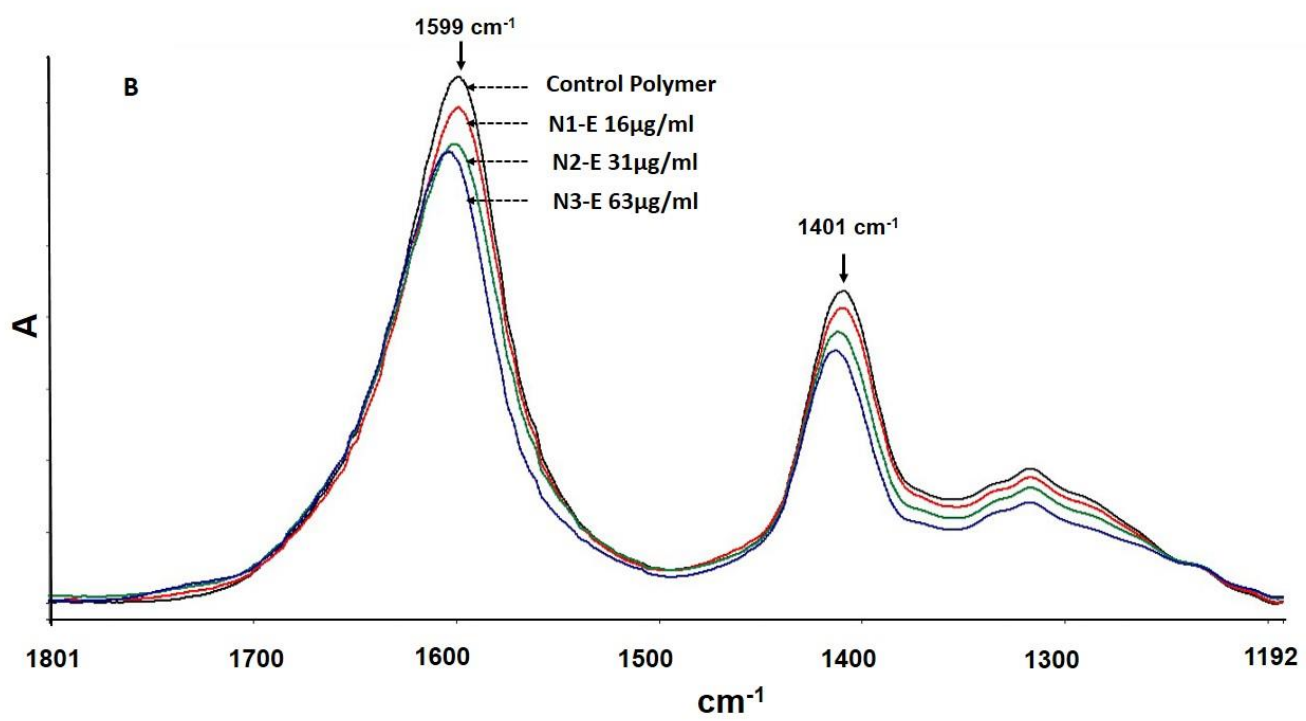


Figure 3.2 B: ATR-FTIR spectra in the wavenumber region between 1801-1192 cm^{-1} .

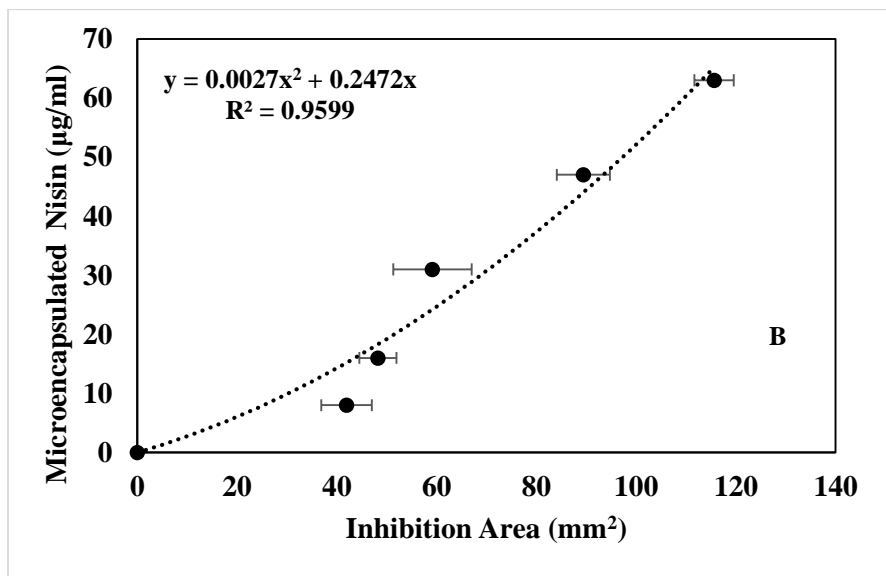
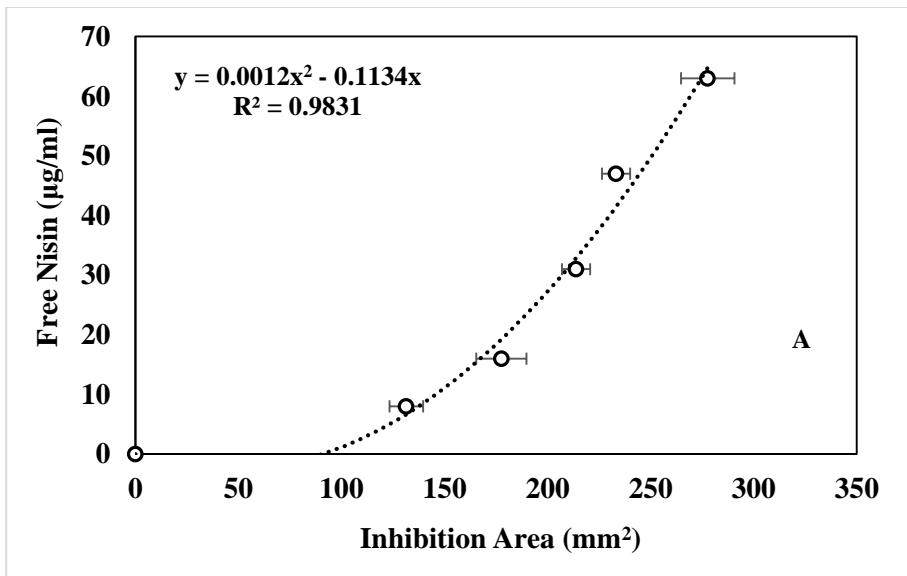


Figure 3.3: Standard curve for A) Free nisin and B) Microencapsulated nisin against *L. monocytogenes* in *in vitro* bioassay.

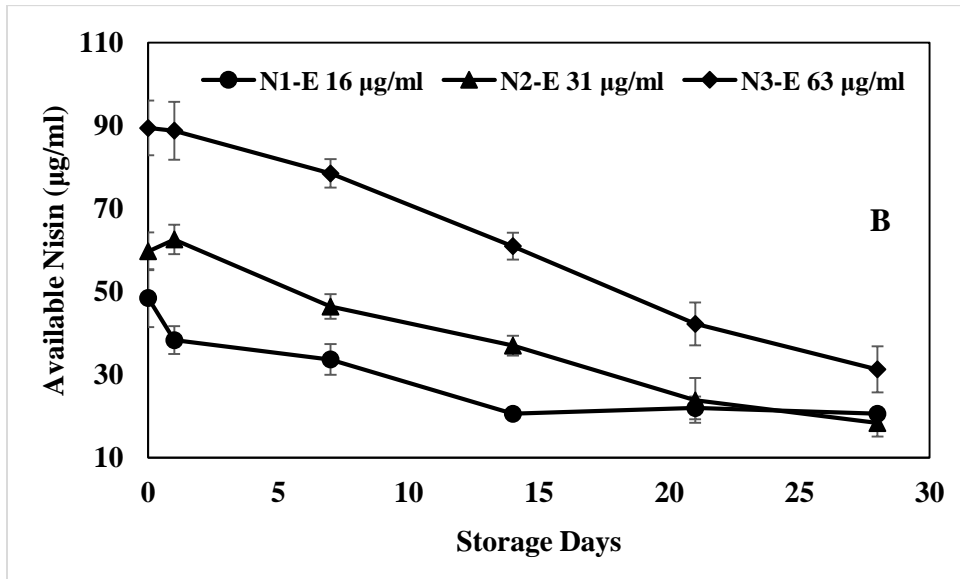
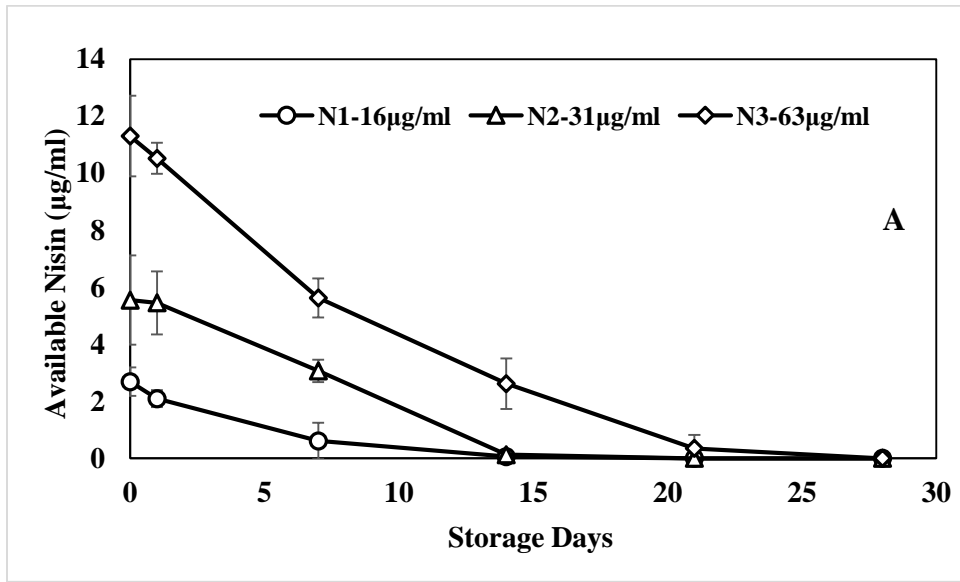
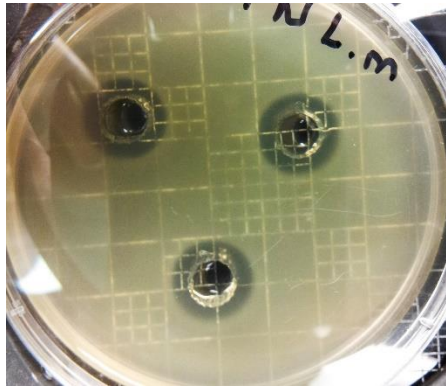
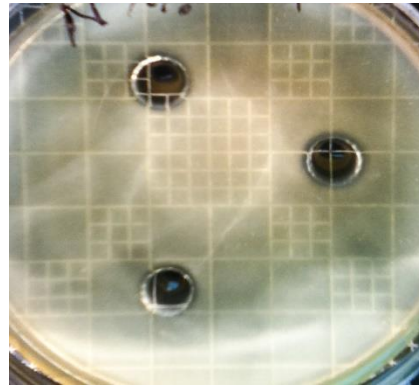


Figure 3.4: Available Nisin Concentration from A) Free nisin and B) Microencapsulated nisin against *L. monocytogenes* during storage at 4° C in *in vitro* BHI-agar deep well model.

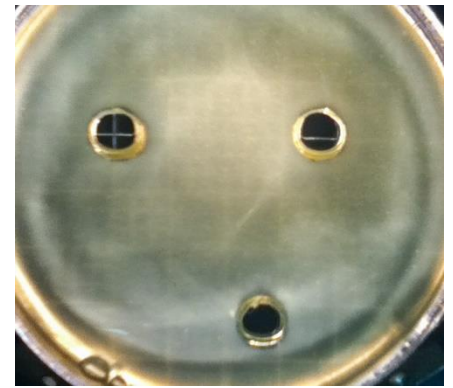
Free Nisin



N3-63 $\mu\text{g/ml}$ (Day-1)

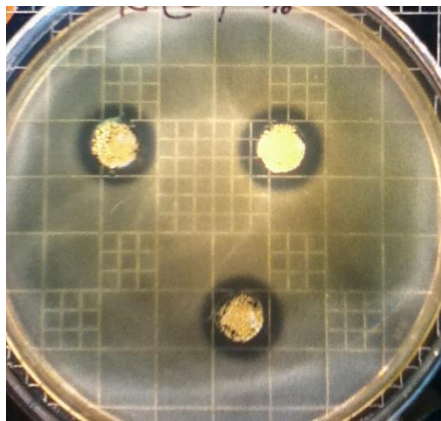


N3-63 $\mu\text{g/ml}$ (Day-14)

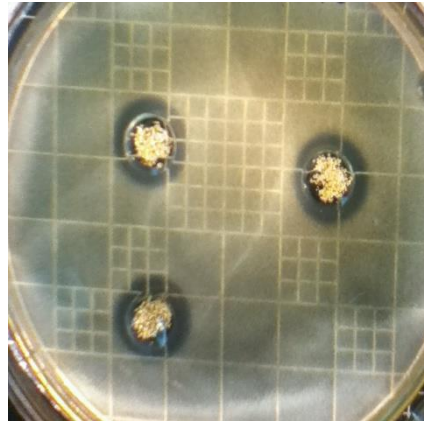


N3-63 $\mu\text{g/ml}$ (Day-28)

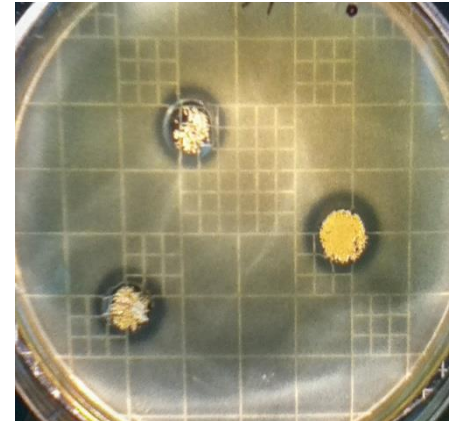
Microencapsulated Nisin



N3-E 63 $\mu\text{g/ml}$ (Day-1)



N3-E 63 $\mu\text{g/ml}$ (Day-14)



N3-E 63 $\mu\text{g/ml}$ (Day-28)

Figure 3.5: The digital photograph of agar diffusion assay against *L. monocytogenes* for free and microencapsulated nisin (N3-63 $\mu\text{g/ml}$) during storage.

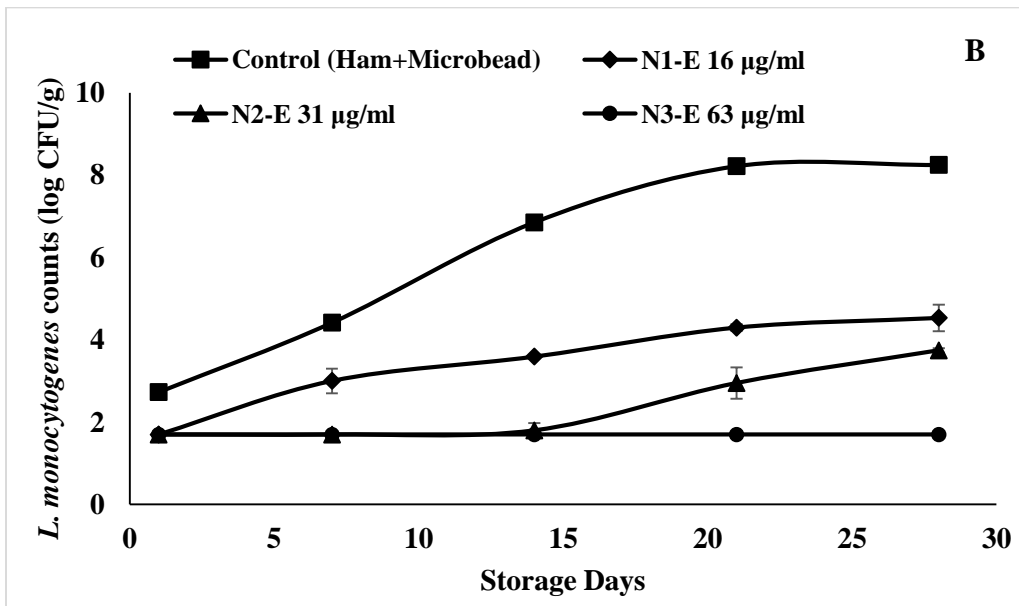
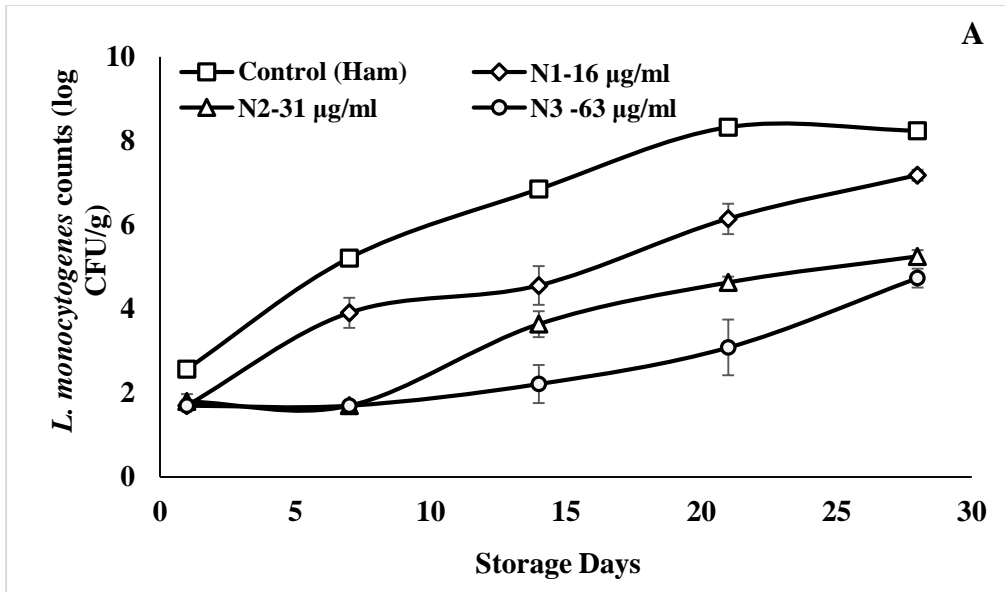


Figure 3.6: Growth of *L. monocytogenes* on vacuum packaged cooked ham slices coated with A) Free nisin and B) microencapsulated nisin during storage at 4° C.

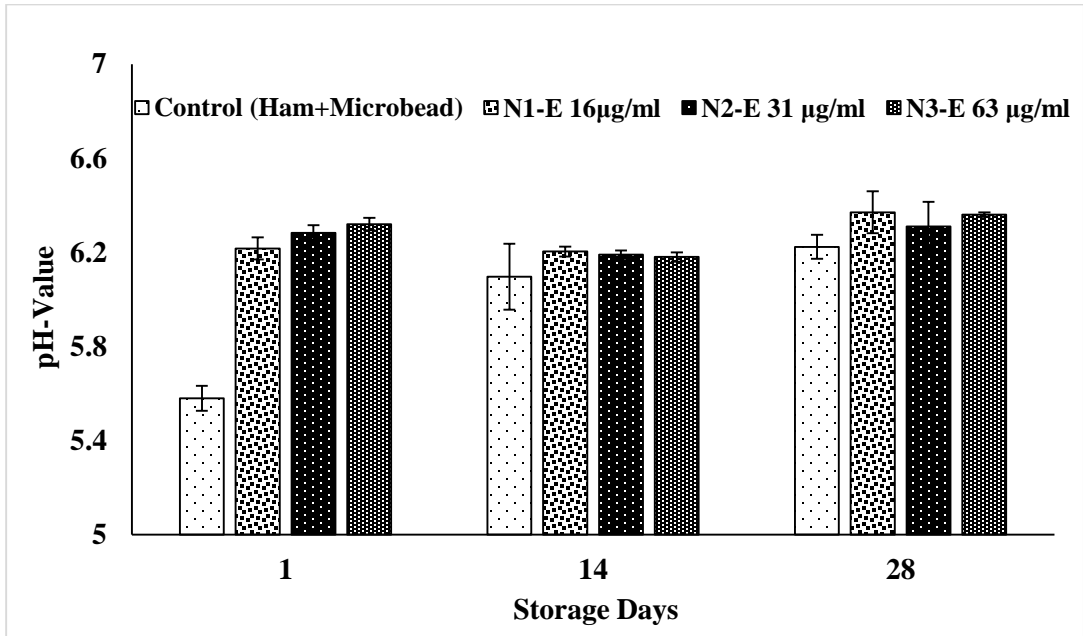
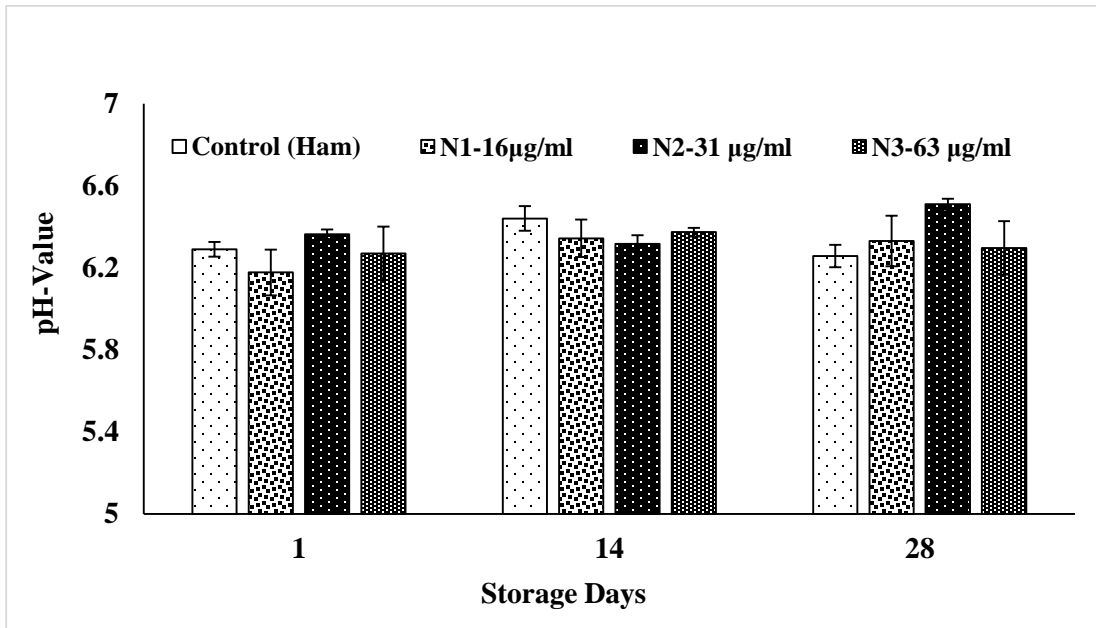


Figure 3.7: pH value of A) free nisin and B) microencapsulated nisin coated RTE cooked ham during storage at 4°C.

Table 3.1 A: Effect of free nisin coating on the colour coordinates L^* , a^* and b^* in RTE cooked ham during storage at 4°C**.

Formulation	L^* -value			a^* -value			b^* -value		
	Day-1	Day-14	Day-28	Day-1	Day-14	Day-28	Day-1	Day-14	Day-28
Control (Ham)	68.36±1.34 ^{aA}	68.15±1.83 ^{aA}	67.18±1.49 ^{aA}	11.18±1.16 ^{abA}	12.35±1.83 ^{bA}	10.21±0.59 ^{aA}	10.40±1.81 ^{aA}	9.50±1.83 ^{aA}	9.98±1.09 ^{aB}
N1-16µg/ml	66.70±2.02 ^{aA}	68.63±2.30 ^{aA}	66.20±0.78 ^{aA}	11.01±1.52 ^{abA}	11.88±1.43 ^{bA}	10.22±0.53 ^{aA}	9.88±1.49 ^{aA}	8.98±1.37 ^{aA}	8.68±0.19 ^{aAB}
N2-31µg/ml	67.55±2.69 ^{abA}	69.13±0.74 ^{bA}	66.53±1.88 ^{aA}	11.41±1.05 ^{bA}	11.35±0.48 ^{bA}	10.23±0.27 ^{aA}	10.81±1.60 ^{bA}	8.00±0.81 ^{aA}	8.67±1.88 ^{aAB}
N3-63µg/ml	66.47±2.39 ^{aA}	68.23±3.18 ^{aA}	65.63±0.55 ^{aA}	10.57±0.88 ^{aA}	10.68±1.13 ^{aA}	10.25±0.24 ^{aA}	9.28±1.67 ^{aA}	7.53±2.07 ^{aA}	8.32±0.64 ^{aA}

** Values are means ± standard deviations. Within each row, means with the same lowercase letter are not significantly different ($P > 0.05$) and within each column, means with the same uppercase letter are not significantly different ($P > 0.05$) as determined by multiple comparison test (Duncan).

Table 3.1 B: Effect of microencapsulated nisin coating on the colour coordinates L^* , a^* and b^* in RTE cooked ham during storage at 4°C^{**} .

Formulation	L^* -value			a^* -value			b^* -value		
	Day-1	Day-14	Day-28	Day-1	Day-14	Day-28	Day-1	Day-14	Day-28
Control (Ham+microbead)	66.35±0.66 ^{aA}	65.58±0.54 ^{aA}	70.20±1.30 ^{bB}	10.11±0.27 ^{bA}	9.45±0.52 ^{aA}	12.47±0.73 ^{cB}	9.51±0.92 ^{bB}	7.58±0.85 ^{aA}	12.73±1.04 ^{cB}
N1-E 16µg/ml	70.22±1.95 ^{bB}	65.62±0.48 ^{aA}	66.15±0.34 ^{aA}	11.70±1.98 ^{bB}	10.18±0.44 ^{aB}	10.12±0.28 ^{aA}	11.10±1.62 ^{cC}	7.12±0.62 ^{aAB}	8.40±0.28 ^{bA}
N2-E 31µg/ml	65.56±1.69 ^{aA}	65.43±1.37 ^{aA}	66.17±0.44 ^{aA}	9.90±0.52 ^{aA}	9.90±0.52 ^{aAB}	10.12±0.41 ^{aA}	6.53±0.59 ^{aA}	6.53±0.59 ^{aB}	7.93±0.40 ^{bA}
N3-E 63µg/ml	67.73±2.24 ^{bA}	64.98±0.65 ^{aA}	66.35±0.61 ^{abA}	10.60±0.83 ^{aAB}	10.08±0.26 ^{aB}	10.25±0.28 ^{aA}	9.81±1.29 ^{bBC}	7.77±0.41 ^{aA}	8.30±0.27 ^{aA}

** Values are means ± standard deviations. Within each row, means with the same lowercase letter are not significantly different ($P > 0.05$) and within each column, means with the same uppercase letter are not significantly different ($P > 0.05$) as determined by multiple comparison test (Duncan).

3.11 General Discussions of the Publication-2

Results demonstrated that microencapsulated nisin microbeads showed its strong antimicrobial activity against *L. monocytogenes* both *in vitro* and *in situ* studies compared to free nisin during storage at 4°C. Alginate-CNC microbeads with nisin (N3-E63µg/ml) completely inhibited the bacterial growth of *L. monocytogenes* during 28 days of storage on RTE meat. Addition of alginate-CNC microbeads with nisin did not show any significant change on color and pH. Thus, this microencapsulation technology with alginate-CNC matrix would be an effective method to improve the acceptance of nisin as an antimicrobial agent for RTE meat applications.

Chapter 4
Publication 3

Synergistic Effect of Gamma (γ)-Irradiation and Microencapsulated Antimicrobials against *Listeria monocytogenes* on Ready-to-Eat (RTE) Meat

Tanzina Huq¹, Khanh Dang Vu¹, Bernard Riedl², Jean Bouchard³ and Monique Lacroix^{1*}

¹Research Laboratories in Sciences Applied to Food, Canadian Irradiation Centre (CIC), INRS-Institut Armand-Frappier, Université du Québec, 531 Boulevard des Prairies, Laval, Québec, H7V 1B7, Canada

²Département des sciences du bois et de la forêt, Faculté de foresterie, géographie et géomatique, Université Laval, Québec, G1V 0A6, Canada

³FPIinnovations, 570 Boulevard St. Jean, Pointe-Claire, Québec, H9R 3J9, Canada

* **Corresponding Author:** Prof. Monique Lacroix

Telephone: +1-450-687-5010; Fax: +1-450-686-5501; **E-mail:** monique.lacroix@iaf.inrs.ca

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4.1 Contributions of the Authors

All the experiments, data analysis and draft have been accomplished by Tanzina Huq with the continuous direction of Prof. Monique Lacroix, my PhD director. During my experiments, Prof. Lacroix has checked and corrected my protocols. Prof. Lacroix has also corrected the main draft and helped me for data analysis. Dr. Khanh Dang Vu also helped me to analyse the data. Both Prof. Bernard Riedl (co-director) and Dr. Jean Bouchard has corrected the draft.

4.2 Specific Objectives of the Publication-3

The specific objectives of the publication-3 were to investigate the synergistic effect of microencapsulated antimicrobials (nisin and essential oils) with γ -irradiation. The lowest concentration of nisin (16 $\mu\text{g/ml}$) (from Publication-2) was combined with *origanum compactum* or *cinnamomum cassia* and was microencapsulated in alginate-CNC microbeads. The antimicrobial activity of these microencapsulated microbeads combined with γ -irradiation was evaluated by in vitro and in situ studies against *L. monocytogens*. The radiosensitivity of *L. monocytogens* was also investigated after addition of antimicrobials microbeads in RTE meat products.

4.3 Résumé

L'huile essentielle d'origan (*Origanum compactum*, 0.025 % w/v), l'huile essentielle de cannelle (*Cinnamomum cassia*, 0.025 % w/v) et de la nisine (0.25% w/v ou 16 µg/ml) ont été utilisés seuls ou en combinaison afin d'évaluer leur efficacité pour inhiber la croissance de *L. monocytogenes* sur du jambon 'prêt-à-manger'. La microencapsulation des formulations antimicrobiennes a été effectuée pour vérifier le potentiel du polymère de protéger l'efficacité antimicrobienne pendant le temps de stockage. Les traitements combinés de la formulation antimicrobienne avec irradiation- γ ont été effectués pour vérifier l'effet synergique contre *L. monocytogenes*. La micro-encapsulation d'huiles, de nisine et un traitement d'irradiation- γ en combinaison a montré un effet antimicrobien synergique lors du stockage de produits de viande 'prêts à consommer'. La cannelle microcapsulée et la nisine en combinaison avec l'irradiation- γ (à 1.5 kGy) ont mené à un taux de croissance de *L. monocytogenes* de 0.03 UFC/g/jour alors que suite à l'ajout de cannelle non encapsulée et de nisine en combinaison avec l'irradiation- γ , la croissance était de 0.17 lnUFC/g/jour. La microencapsulation a également résulté en une amélioration significative de la radiosensitivité de *L. monocytogenes* ($P \leq 0.05$). La microencapsulation de l'origan et d'huile essentielle de cannelle en combinaison avec la nisine a résulté en une radiosensibilisation bactérienne maximale de 2.89 et 5, respectivement, par rapport au témoin.

4.4 Abstract

Oregano essential oil (*origanum compactum*; 0.025% w/v), cinnamon essential oil (*cinnamomum cassia*; 0.025% w/v) and nisin (0.25 % w/v or 16 µg/ml) were used alone or in combination to evaluate their efficiency to inhibit the growth of *Listeria monocytogenes* on ready to eat ham. Microencapsulation of the antimicrobial formulations was done to verify the potential of the polymer to protect the antimicrobial efficiency during storage time. Combined treatments of antimicrobial formulation with γ -irradiation were done to verify the synergistic effect against *L. monocytogenes*. Microencapsulation of essential oils-nisin and γ -irradiation treatment in combination showed synergistic antimicrobial effect during storage on RTE meat products. Microencapsulated cinnamon and nisin in combination with γ -irradiation (at 1.5 kGy) showed 0.03 ln CFU/g/day growth rate of *L. monocytogenes* whereas the growth rate of non-microencapsulated cinnamon and nisin in combination with γ -irradiation was 0.17 ln CFU/g/day. Microencapsulation showed also a significant improvement of *L. monocytogenes* radiosensitivity ($P \leq 0.05$). Microencapsulated oregano and cinnamon essential oil in combination with nisin showed the highest bacterial radiosensitization 2.89 and 5 respectively compared to the control.

Key Words: Gamma irradiation, microencapsulation, essential oils, nisin, *Listeria monocytogenes*

4.5 Introduction

Food production and preservation techniques such as genetic engineering, irradiation on food, modified-atmospheric packaging, food safety are increasing as a public health concern throughout the world. The contamination of food is an important problem, which can necessarily caused by pathogenic microorganisms and deteriorate the quality of food products or cause infection and illness (Lv *et al.*, 2011; Celiktas *et al.*, 2007). The US Public Health Service estimated that 9000 deaths from 6.5 million to 81 million cases of diarrhoeal diseases occur in the US each year due to pathogenic bacteria such as *L. monocytogenes*, *Escherichia coli* and *Salmonella* (Farkas, 1998). Of these, listeriosis accounts for about 2500 cases of illness and approximately \$200 million in monetary loss in the United States annually. *L. monocytogenes* causes 94% hospitalization which results a 15.6% death rate in the US each year. (CDC 2002; Scallan *et al.*, 2011). Listeriosis outbreaks associated with the consumption of RTE meat products have resulted in several illnesses and deaths, and have lead to urgent actions for improving safety of the food products. RTE meat products can be readily contaminated by *L. monocytogenes* during the post-processing stage. The contamination of RTE cooked meat products by *L. monocytogenes* is becoming a major concern because of the long shelf life of these products and are consumed without further processing. *L. monocytogenes* can proliferate on RTE meat products during storage under refrigerated conditions (Zhu *et al.*, 2005). γ -irradiation, one of the post packaging decontamination technologies presently used, is an effective process for reducing or eliminating the growth of *L. monocytogenes* and assure food safety (Thayer and Boyd, 2000; Feliciano *et al.*, 2014). The potential implementation of γ -irradiation on RTE cooked food is mainly based on the fact that it can effectively inactivate the DNA of the pathogenic microorganisms (Farkas, 2006). In 1981, the FAO/IAEA/WHO joint committee accepted γ -irradiation for stored food products. The approval of meat irradiation by the

Food and Drug Administration (FDA, 1997) has made consumers more confident and attracted the interest of industries concerned with food quality and safety. It was stated that, irradiation of food at doses up to 10 kGy introduced no special nutritional problems. Currently, more than 26 countries are using this process on a commercial scale (Stevenson, 1994; Lacroix and Ouattara, 2000; Ouattara *et al.*, 2001; Ha and Kang, 2014). When γ -irradiation is used in combination with antimicrobials, the global efficiency is strengthened through synergistic action and it makes it possible to reduce the irradiation doses without affecting the food quality (Mahrouf *et al.*, 1998). Essential oils (EOs), one of the most widely used natural antimicrobial compounds, are volatile aromatic oily liquids extracted from plants or spices used in food and beverages to improve preservation and sensorial quality (Burt, 2004). Oregano (*Origanum compactum*) and cinnamon (*Cinnamomum cassia*) EOs were used in the present study as antimicrobial compounds against *L. monocytogenes*. The antimicrobial activity of these EOs is due to the main active compounds such as carvacrol (oregano) and trans-cinnamaldehyde (cinnamon) (Oussalah *et al.*, 2007a; Oussalah *et al.*, 2007b). EOs have a GRAS (generally recognised as safe) status but the acceptable concentration is limited due to their organoleptical criteria (Lambert *et al.*, 2001; Dussault *et al.*, 2014). Bacteriocins such as nisin are a class of antimicrobial compounds produced by several strains of *Lactococcus lactis* and recognized as GRAS by the United States Food and Drug Administration (FDA) as stated in the Code of Federal Regulations (CFR section 184.1538) (Millette *et al.*, 2007). It is a cationic and amphiphilic polypeptide composed of 34 amino acids, with antibacterial activity against many Gram-positive bacteria such as *L. monocytogenes* (Chen and Hoover, 2003). EOs and nisin both are very unstable in direct contact with the food system (Brasil *et al.*, 2012; Rose *et al.*, 1999) which could be minimized by microencapsulation of these two compounds. Microencapsulation is a technology that allows sensitive ingredients (such as EOs

and nisin) to be physically entrapped in a homogeneous or heterogeneous biopolymeric matrix aiming at their maximum protection during long term storage (Beirao da Costa *et al.*, 2012; Marcos *et al.*, 2007).

Previous studies revealed that edible coatings could preserve the biological activity of the encapsulated antimicrobials during storage and also ensure proper dispersion of the active compounds on the food surfaces which is the typical point of entry of pathogens (Lacroix and Ouattara, 2000; Coma, 2008). The choice of biopolymers is an important step for the success of the microencapsulation processes. An alginate-Cellulose nanocrystal (CNC) matrix was used in this current investigation due to its good physicochemical properties which was demonstrated by our previous study (Huq *et al.*, 2012b). The use of CNC improved the physicochemical characteristics of alginate and it has also been demonstrated that CNC has the property to stabilize the emulsion (Kalashnikova *et al.*, 2012) as EOs make emulsions with the alginate-CNC matrix. Microencapsulation of antimicrobials in alginate-CNC matrix is an innovative approach to protect the active compounds on RTE meat products during storage. The synergy between microencapsulated antimicrobials and irradiation has already been reported for vegetable food products (Gomes *et al.*, 2011; Severino *et al.*, 2014). The aim of this study was to evaluate the potential of the microencapsulation based on alginate-CNC to protect the antimicrobial activity of the natural antimicrobial compounds during storage by *in vitro* and *in situ* studies. The synergistic effect of γ -irradiation and microencapsulation of antimicrobials compounds was also investigated on RTE cooked ham for radio-sensitization of *L. monocytogenes*.

4.6 Materials and Methods

4.6.1 Materials

Sodium alginate (alginic acid sodium salt from brown algae, guluronic acid (Eq) or glucuronic (Ax) content ~ 65 - 70%; mannuronic acid content ~5 - 35%), calcium chloride (granules) and lactic acid were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). CNC was supplied from FP Innovations which was produced in FP Innovations pilot plant (Pointe-Claire, QC, Canada) from a commercial bleached softwood kraft pulp according to a procedure modified from the literature (Dong et al., 1998). Nisin (Niprosin™, purity 2.5%, 77.5% salt and 20% vegetable protein, Profood, Naperville, IL, USA) was purchased from Pro-food International Inc.. EOs such as *Origanum compactum* and *Cinnamomum cassia* were purchased from Robert et Fils (Montreal, QC, Canada). ALCOLEC® PC 75 (lecithin) was obtained from American Lecithin Company, (Oxford, CT, USA). Brain heart infusion (BHI) broth and Palcam agar were purchased from Alpha Biosciences Inc. (Baltimore, MD, USA). Preservative salts such as sodium chloride, triphosphate, erythorbate and nitrite salt were delivered from BSA Food Ingredients (St-Leonard, QC, Canada). Ground lean pork meat was purchased from a local grocery store (IGA, Laval, QC, Canada).

4.6.2 Preparation of Nisin Solution

Nisin (0.25% w/v) was prepared by dispersing Niprosin™ powder in 100mL CaCl₂ (0.01 M) solution and the pH of the nisin-CaCl₂ solution was adjusted to 3-3.5 by diluted lactic acid. Nisin-CaCl₂ solution was stirred overnight for proper mixing. After stirring overnight, nisin-CaCl₂ solution was centrifuged for 15 min at 3500×g, 4°C to remove the undissolved particles and collected the nisin-CaCl₂ supernatant.

4.6.3 Microencapsulation of Nisin and EOs

An aqueous suspension was prepared by dissolving 3% (w/v) of alginate in deionized water for 24hr under magnetic stirring. A 1% (w/v) CNC suspension was prepared by dispersing spray dried CNC powder in deionized water under magnetic stirring. Then, the CNC suspension was subjected to ultra-sonication (QSonica Q-500, Misonix, Qsonica, LLC, Newtown, CT, USA) at 1000 J/g of CNC. A 5 % (w/w) CNC from 1 % CNC suspension (according to wt% of alginate) was homogenized using an Ultra-Turrax TP18/1059 homogenizer (Janke and Kunkel, Staufen, Germany) at 23°C and 25000 rpm for 1 min. The Alginate-CNC suspension was emulsified with *Origanum compactum* and *Cinnamomum cassia* (0.025% w/v) using lecithin (0.25% w/v). Nisin-CaCl₂ solution was mixed with EOs containing emulsified alginate-CNC suspension according to alginate-CNC-EOs: nisin-CaCl₂: 75:25 (Rajaonarivony *et al.*, 1993). The final concentration of EOs in the alginate-CNC microbeads was 250 µg/ml and for nisin, it was calculated according to the activity of pure nisin (2.5%) which was 16 µg/ml in alginate-CNC microbead. The free antimicrobials were also verified in order to evaluate the effectiveness of microencapsulation. The treatments were denoted as follows

OR: free *Origanum compactum*; OR(E): microencapsulated *Origanum compactum*; CN: free *Cinnamomum cassia*; CN(E): microencapsulated *Cinnamomum cassia*; N: free nisin; N(E): microencapsulated nisin; OR+N: free *Origanum compactum*-nisin; OR+N (E): microencapsulated *Origanum compactum*-nisin; CN+N: free *Cinnamomum cassia*-nisin; CN+N (E): microencapsulated *Cinnamomum cassia*-nisin

4.6.4 Bacterial Culture

Five strains of *L. monocytogenes* HPB (Health Canada, Health Products and Food Branch, HPB, Ottawa, Canada) were used in this experiment. All bacterial strains were isolated from

contaminated food samples: HPB 2558 1/2b from beef hot dogs, HPB 2818 1/2a from homemade salami, HPB 1043 1/2a from raw turkey, 2569 1/2a from cooked cured sliced turkey and HPB 2371 1/b from raw turkey. The microorganisms were kept frozen at -80°C in BHI broth containing glycerol (10% v/v). Before use, the stock cultures were resuscitated through 2 consecutive 24 h growth periods in BHI at 37°C to obtain the working cultures containing approximately 10⁹ CFU/mL.

4.6.5 BHI-Agar Deep-Well Model to Evaluate Depletion Activity of EOs and Nisin

To prepare the BHI-agar deep-well model for each of the formulations (free or microencapsulated) depletion test, a solution of BHI solids (3.7%) and agar (1.0%) solution was autoclaved for 20 min at 121°C. The hot solution (225mL) was poured into a 600-mL beaker to a height of 40mm. After gel solidification, four wells (from gel surface to bottom) were made in each beaker using a 7.0-mm pipet tip. Subsequently, 1.0mL of each formulation preparation was added to each well. Immediately after loading at day 0 and after 1, 3, 7, 14 and 21 days of storage at 4°C, a 100- μ L aliquot of each formulation preparation was transferred from the well to a bioassay plate to determine the available antimicrobial content. For γ -irradiation, all formulations were irradiated at 1.5kGy and stored in BHI-agar well and was treated indentially to the non-irradiated samples to verify the effect of γ -irradiation on the available antimicrobial contents (free and microencapsulated) during storage (Bi *et al.*, 2011).

4.6.6 EOs and Nisin Bioassay against L. monocytogenes

Available free or microencapsulated antimicrobial content, either for those freshly prepared or stored in BHI-agar deep-well, was determined as described by Pongtharangkul and Demirci (2004) with some modifications. The γ -irradiated preparations were also checked using the same procedure. BHI-agar with Tween 20 (1.0%) was prepared and autoclaved. After cooling, the

solutions were inoculated with 1.0% (v/v) BHI broth containing *L. monocytogenes* (10^6 CFU/mL). 25 mL of inoculated broth was added to each Petri dish plate (95mm×15mm) and allowed to solidify. Thereafter, holes 7.0mm in diameter were made and 100- μ L of nisin and essential oils preparation were added to each agar well. The plates were incubated for 24 h at a temperature of 37°C and the inhibition zone (mm) was measured to determine the activity against *L. monocytogenes*.

To evaluate the available antimicrobial concentration, a standard curve was prepared with Chloramphenicol (broad spectrum antibiotic). Chloramphenicol is widely used for antimicrobial drug standardization (Hammett-Stabler & Johns, 1998). Regression analysis of the standard curve was performed and the inhibition zone (mm) of the antimicrobials was correlated of that standard equation. The available antimicrobial concentration was expressed as Chloramphenicol equivalent (mg/ml CAM).

4.6.7 Preparation of Ham Samples

Ground lean pork was cooked with the following preservatives: sodium chloride (1.5%), tripolyphosphate (0.43%), sodium erythorbate (750 ppm) and sodium nitrite (50 ppm) salt for about 1hr at 162.7° C in a cooking oven. After cooking, the ham was removed from the oven and placed at 4° C for 24 hr. Then the cooked ham was sliced (20g) and 8 ml of free or microencapsulated antimicrobials was coated on each ham slice. The coated cooked ham slice was then inoculated with ~ 5 to 6 log CFU/g *L. monocytogenes* and vacuum packaged within 24hr of production.

4.6.8 Irradiation Treatment

Inoculated cooked ham samples were irradiated at the Canadian Irradiation Center under refrigerated conditions at 1.5 kGy. The UC-15A underwater calibrator (MDS Nordion) was

equipped with a ^{60}Co source that provided a dose rate of 17.698 kGy/h. Samples were stored at 4°C and were analyzed at each storage interval (1, 7, 14, 21, 28 and 35 days).

4.6.9 Microbiological Analysis

Each ham sample (20 g) was homogenized for 2 min in sterile peptone water (0.1%, wt/vol; Difco, Becton Dickinson) in a Lab-blender 400 stomacher (Seward Medical, London, UK). From this homogenate, appropriate serial dilutions were prepared in 0.1% peptone, and 100 µl of each dilution was spread plated on Palcam which was supplemented with acriflavine (5 mg/mL), polymyxin B (10 mg/mL) and ceftazidime (8 mg/mL) for the selective enumeration of *L. monocytogenes*. The plates were incubated at 37 °C for 48 h. Following incubation, the colony forming units (CFU) were counted using a magnifier. The detection limit for all microbial analyses was 50 CFU/g of meat. All bacterial counts were transformed to logarithmic (log₁₀) values.

4.6.10 Bacterial growth rate

The growth rate (μ) of *L. monocytogenes* in the RTE meat samples can be described according to Eq. 1 over duration of 35 days.

$$\mu = [\ln (X_2/ X_1)]/[T_2-T_1] \quad (1)$$

where, X_2 and X_1 are population of *L. monocytogenes* at day T_2 and T_1 , respectively. The growth rate constant is presented as μ (ln CFU/day/g). At first, CFU count (obtained during the *in situ* test) were transformed into natural logarithmic (ln) values and μ was determined by calculating the slope from the linear portion of the each bacterial count (ln CFU/g) vs. storage (days) curve.

4.6.11 Radiosensitization Analysis

Inoculated cooked ham samples were exposed to 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75 and 2 kGy. D_{10} values were calculated (defined as the radiation dose required to reduce one log CFU of population) from the following linear regression of the bacterial destruction kinetics,

$$\text{Log } N_t / N_0 = -kt \quad (2)$$

Where, N_0 is the initial bacterial count in CFU/g, N_t is the bacterial count in CFU/g at different irradiation doses and k is the bacterial destruction rate. Bacterial counts (CFU/g) were plotted against different irradiation doses and the reciprocal of the slope of the trend line was extracted from the plot to calculate D_{10} value. The degree of radiation sensitivity (RS) was determined by the following equation:

$$RS = \frac{D_{10}(\text{control})}{D_{10}(\text{treatment})} \quad (3)$$

Where, $D_{10}(\text{control})$ is the D_{10} -value without antimicrobial and $D_{10}(\text{treatment})$ is the D_{10} -value of the samples treated with free and microencapsulated antimicrobials (Severino *et al.*, 2014; Turgis *et al.*, 2012).

4.6.12 Statistical Analysis

To validate the results obtained during different experimental procedure, each analysis was carried out in triplicate in a completely randomized experimental design. An analysis of variance (ANOVA) and multiple comparison tests of Duncan's were used to compare all the results. Differences between means were considered significant when the confidence interval was smaller than 5% ($P \leq 0.05$). The analysis was performed by the PASW Statistics 19 software (IBM Corporation, Somers, NY, USA).

4.7 Results and Discussions

4.7.1 Effect of γ -irradiation on Available Antimicrobial Content (Free or Microencapsulated) during Storage: in vitro

Figure 4.2 represents the effect of γ -irradiation on available free or microencapsulated antimicrobials, mg/ml CAM (oregano and nisin). A 2-order polynomial chloramphenicol “standard curve” (**Figure 4.1**) was used to correlate the inhibition zone (mm) of the available antimicrobials. Initially at day 0, the available content of OR was 0.60 mg/ml CAM, but it decreased to 0.05 mg/ml CAM within first the 7 days of storage and continued to decrease in the later stages. Whereas, microencapsulated OR (E) showed more available content after 7 days of storage and it was 0.20 mg/ml CAM. N and N (E) exhibited the similar decrease in available content during storage. Results revealed that microencapsulated combined antimicrobial OR+N (E) showed better activity compared to free combined antimicrobials OR+N during storage. After 7 days of storage, the available content for OR+N (E) was 0.38 mg/ml CAM whereas the available content for OR+N was 0.18 mg/ml CAM. The microencapsulated OR+N (E) showed its available content up to 21 days of storage but free OR+N almost lost its activity after 21 days of storage. Microencapsulation also showed a protection for antimicrobials during γ -irradiation. After 7 days of storage, the available content for OR+N (E)- γ was 0.26 mg/ml CAM whereas the available content for OR+N - γ was 0.03 mg/ml CAM.

Figure 4.3 represents the effect of γ -irradiation on available free or microencapsulated antimicrobials, mg/ml CAM (cinnamon and nisin). After 7 days of storage, microencapsulated CN (E) had more available content than the non-microencapsulated CN. Results showed that the available content for CN (E) and CN was 0.27 and 0.03 mg/ml CAM. CN+N (E) showed better protection of antimicrobials than the CN+N. After 14 days of storage, the available content for

CN+N (E) was 0.16 mg/ml CAM whereas CN+N lost its activity. In this formulation, γ -irradiation showed the similar activity as oregano-nisin formulation. After 7 days of storage, the available content for CN+N (E)- γ was 0.25 mg/ml CAM but CN+N- γ lost its activity. This study demonstrated that free antimicrobials lost their activity within a very short time after γ -irradiation that microencapsulation could protect initially. But γ -irradiated microencapsulated formulations did not show the activity more than 14 days. Huq *et al.* (2012a) reported that alginate could be degraded during gamma irradiation due to the formation of hydroxyl radicals by radiolysis of water. These free radicals can reduce the activity of antimicrobials even though free antimicrobials are directly exposed to gamma irradiation and lost their antimicrobial activity more than microencapsulated formulations. Further, *in situ* studies were done to further clarify these results.

4.7.2 Synergistic Effect of Microencapsulated Combined Antimicrobials and γ -irradiation (at 1.5 kGy) during Storage: in situ

4.7.2.1 OR+N (E) and γ -irradiation (at 1.5 kGy) during Storage

Figure 4.4 represents the effect of microencapsulation of oregano and nisin on RTE meat during storage at 4°C. Formulations C and C (E) had higher *L. monocytogenes* counts after 35 days of storage at 4°C and it was 8.44 and 8.32 log CFU/g, respectively. The growth rate for C and C (E) was 0.23 and 0.24 ln CFU/g/day, respectively (**Table 4.1**). Similarly, OR and N showed *L. monocytogenes* counts of 8.02 and 7.79 log CFU/g, respectively, after 35 days of storage at 4°C. Combination of OR+N increased the antimicrobial activity during storage and *L. monocytogenes* counts was 6.87 log CFU/g after 35 days of storage for OR+N. Microencapsulated OR (E) and N (E) showed *L. monocytogenes* counts 7.91 and 7.14 log CFU/g after 35 days of storage. Formulation OR+N (E) enhanced the antimicrobial activity during storage and results showed that

after 35 days of storage, OR+N (E) reduced ($P \leq 0.05$) *L. monocytogenes* counts by 1.07 log CFU/g.

Figure 4.5 shows the synergistic effect of microencapsulated antimicrobials with γ -irradiation. The bacterial counts for C- γ and C (E)- γ was found to be 2.84 and 2.94 log CFU/g, respectively, at 1 day of storage which represented the immediate effect of irradiation. After 35 days of storage, the bacterial counts for C- γ and C (E)- γ reached a level of 6.71 and 6.47 log CFU/g, respectively. Both OR+N- γ and OR+N (E)- γ showed a lag phase of bacterial growth up to 7 days of storage. Lag phase could be defined as the initial growth phase, during which cell number remains relatively constant prior to rapid growth which might be occurred in both growth and inactivation processes (Swinnen et al., 2004). OR+N- γ and OR+N (E)- γ showed lower bacterial counts and a level of 4.31 and 3.02 log CFU/g was found after 35 days of storage respectively. However, both OR+N- γ and OR+N (E)- γ showed the bacterial growth rate of 0.14 and 0.13 ln CFU/g/day. Thus, microencapsulated combined antimicrobials and γ -irradiation [OR+N (E)- γ] reduced the bacterial growth rate by 32% compared to microencapsulated combined antimicrobials without irradiation [OR+N (E)].

4.7.2.2 CN+N (E) and γ -irradiation (at 1.5 kGy) during Storage

Both microencapsulated and free *Cinnamomum cassia* and nisin showed similar bacterial counts during 35 days of storage at 4°C (**Figure 4.6**). Whereas during storage, the combination of antimicrobials CN+N reduced the bacterial counts by 3.03 log CFU/g compared to C after 35 days of storage. The microencapsulated antimicrobials CN+N (E) samples showed a 4.04 log CFU/g reduction as compared to C (E) and also showed the bacterial growth rate 0.05 ln CFU/g/day.

After γ -irradiation treatment (**Figure 4.7**), both CN+N- γ and CN+N (E)- γ showed bacterial counts below the detection limit (≤ 50 CFU/g) at day 1. Therefore, after 35 days of storage, CN+N- γ and

CN+N (E)- γ exhibited bacterial counts of 3.96 and 1.80 log CFU/g, respectively. γ -irradiation increased the lag phase of CN+N (E)- γ up to 28 days but CN+N- γ showed only a 7 days lag phase. The bacterial growth rate for CN+N- γ and CN+N (E)- γ was 0.17 and 0.03 ln CFU/g/day whereas C- γ (control ham) and C (E)- γ (control with microbeads without antimicrobials) showed a bacterial growth rate of 0.29 and 0.26 ln CFU/g/day. Hence, microencapsulation and irradiation allowed a synergistic effect on the antimicrobial activity during storage.

Abdollahzadeh *et al.* (2014) reported that a combination of thyme EO and nisin reduced the *L. monocytogenes* counts by 1.86 log CFU/g after 4 days of cold storage which remained unchanged up to 12 days of storage in minced fish meat which is supported by our present findings. The antimicrobial activity of EOs are predominately due to their active phenolic components. Turgis *et al.* (2012b) also demonstrated the antimicrobial activity of *Origanum compactum* and *Cinnamomum cassia* in combination with nisin against *L. monocytogenes* by *in vitro* study. According to manufacturer's specifications (Robert et Fils), *O. compactum* (OR) and *C. cassia* (CN) are a mixture of carvacrol (46.37%), thymol (13.70%), γ -terpinene (12.32%), p-cymene (13.33%) and cinnamaldehyde (64.59%), methoxy cinnamaldehyde (20.82%), respectively. Solomakos *et al.* (2008) reported that thymol and carvacrol caused ion leakage on cell membranes due to the phenolic compounds present in thyme EO. The authors also found that thyme EO in combination with nisin slowed the *L. monocytogenes* growth 2.3 log CFU/g after 12 days of storage in mined beef meat. According to Ayari *et al.* (2010), the addition of carvacrol in broth and irradiation treatment initiated the disintegration of the outer membrane and disruption of the cytoplasmic membrane permeability of the cell and helped to reduce intracellular ATP (energy), making it nearly impossible for the cell to repair the damage. Moleyar and Narasimham (1986) and Oussalah *et al.* (2006) showed that biological processes involving electron transfer could be

interfered by cinnamaldehyde which reacts with vital nitrogen components such as proteins and nucleic acids, thus inhibiting the growth of the microorganisms. Nisin, consisting of 34 amino acids, is mainly exhibited its antimicrobial properties against gram positive bacteria such as *L. monocytogenes*. The generally accepted mode of action for nisin on vegetative cells involves the formation of pores in the cytoplasmic membrane of target cells leading to the efflux of essential small cytoplasmic components, such as amino acids, potassium ions and ATP (Prombutara *et al.*, 2012).

The combination of EOs and nisin was found to be most effective antimicrobial which is thought to be due to the multiple attack of the cell membrane by individual active components like carvacrol, thymol, trans-cinnamaldehyde and mixture of amino acids. Pol and Smid, (1999) investigated the combination between carvacrol and nisin against *L. monocytogenes* and *Bacillus cereus*. They declared that carvacrol might enhance the antibacterial activity of nisin by lengthening the lifetime of pores created by nisin, or by increasing the number or size of the pores formed which altered the permeability of the membrane, proton motive force, efflux of amino acid and the pH gradient of bacteria. Thus combination of EOs and nisin lead to a reduction of viable cell more than individual EOs and nisin. But nisin was found to be very unstable in contact with food components. Rose *et al.* (1999) reported that direct addition of nisin may also be inactivated by proteolytic enzymes of meat components such as Glutathione-S-transferase(GTS). More than 50% of free nisin was inactivated by RTE meat components after 4 days of storage (Stergiou *et al.*, 2006). EOs could also be lose their activity by volatilization of the active components or its degradation due to the temperature variance and oxidation with the food system (Soliman *et al.*, 2013). It was reported that high levels of fat and/or protein in foodstuffs protect the bacteria from the action of the EO in some way. When EOs come in direct contact with the fat or protein

components of RTE meat, they could readily react with fat by oxidation and lose their activity during storage (Burt, 2004). Microencapsulation can resolve these problems by preventing a direct contact of the antimicrobials with food system. Alginate-CNC microbeads were found to be one of the best biopolymeric matrices due to their non-toxic and edible properties (Alexandrescu *et al.*, 2013; Goh *et al.*, 2012). A synergistic effect was found by a combination of microencapsulated EOs and nisin combined with γ -irradiation which would be a high impact technology to improve the RTE meat market with respect to pathogenic contamination. Further studies need to be done to understand the antibacterial mechanism of microencapsulated antimicrobials with γ -irradiation.

4.7.3 Effect of Microencapsulated Antimicrobials on Radiosensitization of *L. monocytogenes*

The influence of microencapsulated antimicrobials on the radiosensitization of *L. monocytogenes* has been presented in **Table 4.2**. Results demonstrated that a combination of antimicrobial (EOs and nisin) coatings enhanced the radiosensitivity (RS) of *L. monocytogenes* on RTE cooked ham compared to individual antimicrobial coatings. Microencapsulation of combined antimicrobials was found to be the most effective formulation for the radiosensitization of *L. monocytogenes* on RTE meat. In current study, D_{10} -values of 0.54 and 0.55 kGy were observed for control ham (C) and control ham with microbeads, C (E) (**Table 4.2**). Both free and microencapsulated antimicrobial formulation coated ham reduced ($P \leq 0.05$) the D_{10} -values compared to control ham without and with microbeads. Caillet *et al.* (2006) reported that Spanish oregano (*Corydothymus capitatus*) and Chinese cinnamon (*Cinnamomum cassia*) EO coatings without polymers on ready-to-use carrots increased the RS of *L. monocytogenes* by 3.05 and by 3.86-fold compared to control samples. Whereas our study revealed that free *Origanum compactum* (OR) and *Cinnamomum cassia* (CN) coating on RTE ham increased the RS by 1.20 and by 1.86 fold compared to control which was probably due to the difference of the food system. Turgis *et al.* (2012a) showed that

addition of nisin enhanced the RS by 1.23-fold of *L. monocytogenes* on sausage meat. Similarly, our result using nisin (N) coatings also improved 1.93-fold RS of *L. monocytogenes* on RTE meat. Previous studies showed that combined antimicrobial and γ -irradiation treatments have a synergistic effect on the radiosensitization of *L. monocytogenes* (Caillet *et al.*, 2006; Takala *et al.*, 2011b). Takala *et al.* (2011a) reported that a combination of rosemary EO and organic acid encapsulated in methylcellulose amplified by 1.34-fold the RS of *L. monocytogenes* on ready to eat broccoli. Microencapsulation of combined antimicrobials (EOs and nisin) significantly ($P \leq 0.05$) increased the radiosensitivity of *L. monocytogenes* on a RTE food system which was demonstrated by this present study. Combined microencapsulated antimicrobials OR+N (E) and CN+N (E) coated ham showed the lowest D_{10} -values (0.19 and 0.11 kGy, respectively) compared to free combined antimicrobials OR+N and CN+N (0.26 and 0.23 kGy, respectively). Thus, OR+N (E) and CN+N (E) exhibited higher radiosensitivities (RS) of 2.89 and 5.00 fold, respectively compared to control ham. Results revealed that OR+N (E) and CN+N (E) increased by 39 and 113% the bacterial radiosensitivity (RS) compared to OR+N and CN+N which showed a significant ($P \leq 0.05$) improvement of the radiosensitization on RTE meat. Similar data were also obtained in previous studies using EOS mixed in ground beef before irradiation treatment (Borsa *et al.*, 2004; Zhu *et al.*, 2009). Bacterial radiosensitization is a recognised biological phenomenon which has been found to be useful in meat application (Lacroix *et al.*, 2000). γ -irradiation is one type of ionizing radiation that destroys the microorganisms by direct breakdown of chemical bonds in bacterial DNA or by the indirect effects of reactive oxygen species produced by the radiolysis of water on cell membranes and chromosomes (Ward, 1991). These changes may facilitate the contact between antimicrobial molecules and cell membranes and thus increase the inhibitory effects of these molecules against *L. monocytogenes*. Therefore, ionizing radiation can enhance

its activity in the presence of antimicrobial compounds such as essential oils and nisin. This hypothesis is also supported by the findings of Mahrouf *et al.* (2003), who treated poultry meats with combined irradiation and marination with rosemary, thyme and lemon juice and due to the activity of EOs, they obtained a significant increase in bacterial radiosensitization.

4.8 Conclusion

Microencapsulation of EOs and nisin showed a synergistic anti listerial effect with γ -irradiation on RTE meat products. The FDA (US Food and Drug Administration) maintains a policy of “zero-tolerance” for *L. monocytogenes* in RTE meat products (U.S. Food and Drug Administration, 2003). Our findings confirmed that CN+N (E)- γ showed a strong inhibitory effect up to 28 days and the bacterial count was below detection levels. Furthermore, microencapsulation technology with irradiation could be an advanced process to improve the food safety for the RTE meat market.

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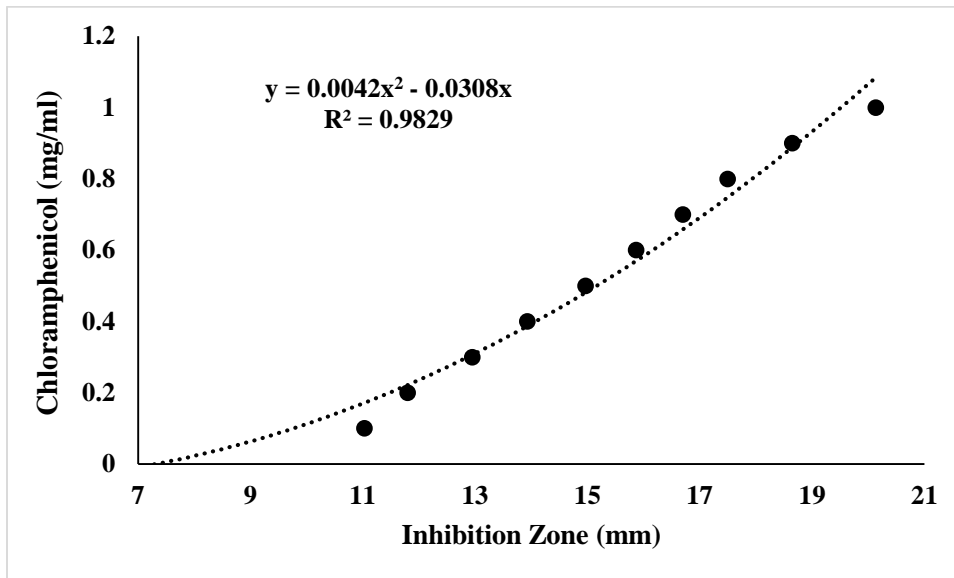


Figure 4.1: Standard curve of Chloramphenicol against *L. monocytogenes* in *in vitro* bioassay.

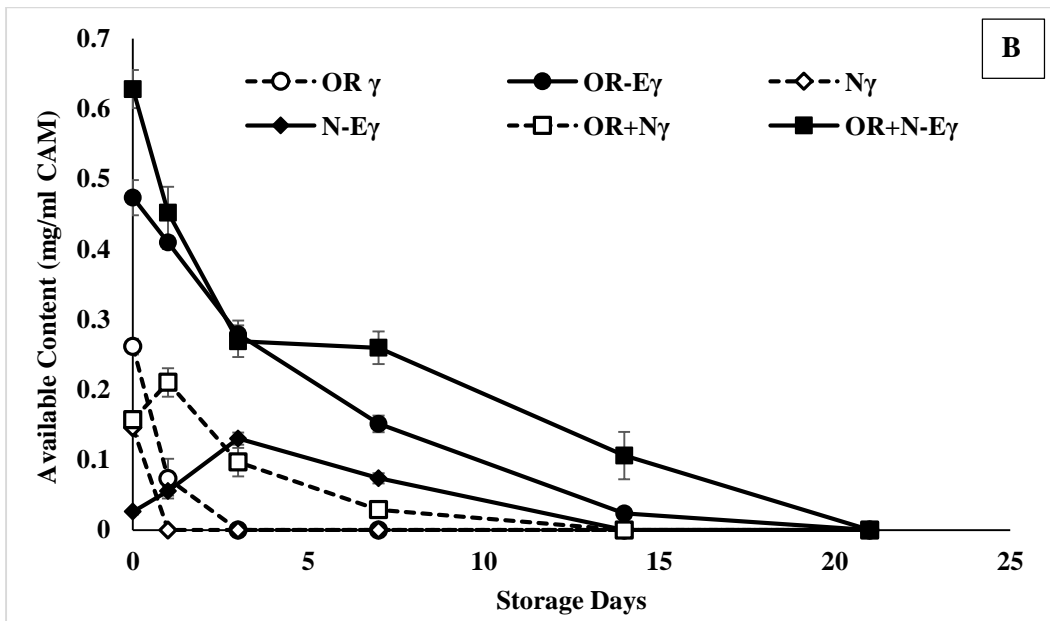
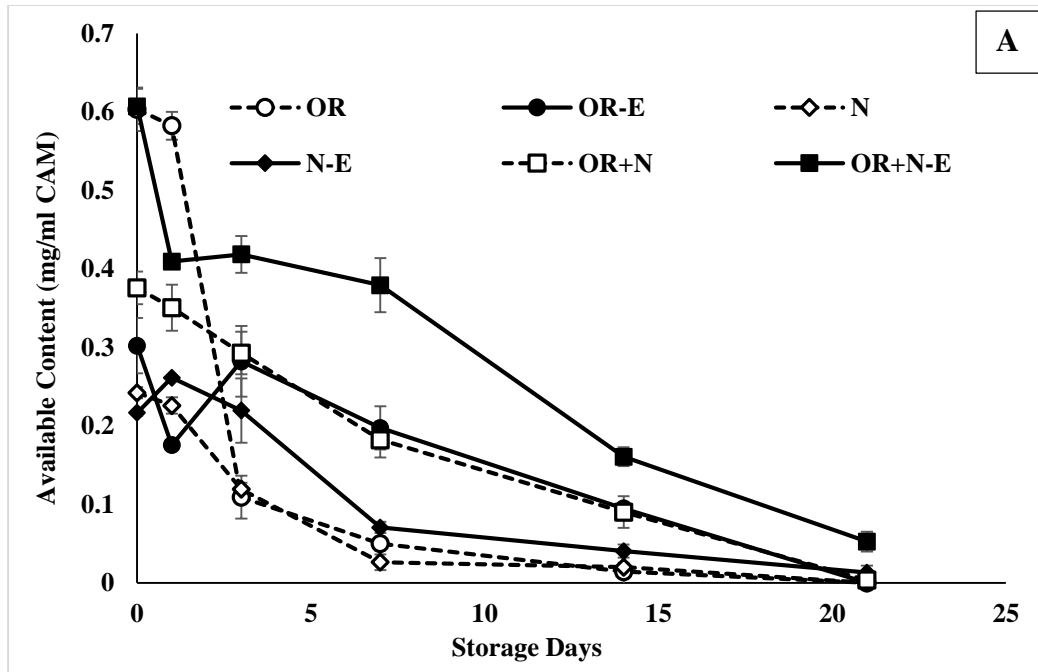


Figure 4.2: Effect of γ -irradiation on available free or microencapsulated antimicrobials, mg/ml CAM (*Origanum compactum*) against *L. monocytogenes* during storage at 4° C in *in vitro* BHI-agar deep well model. A) without irradiation and B) with irradiation.

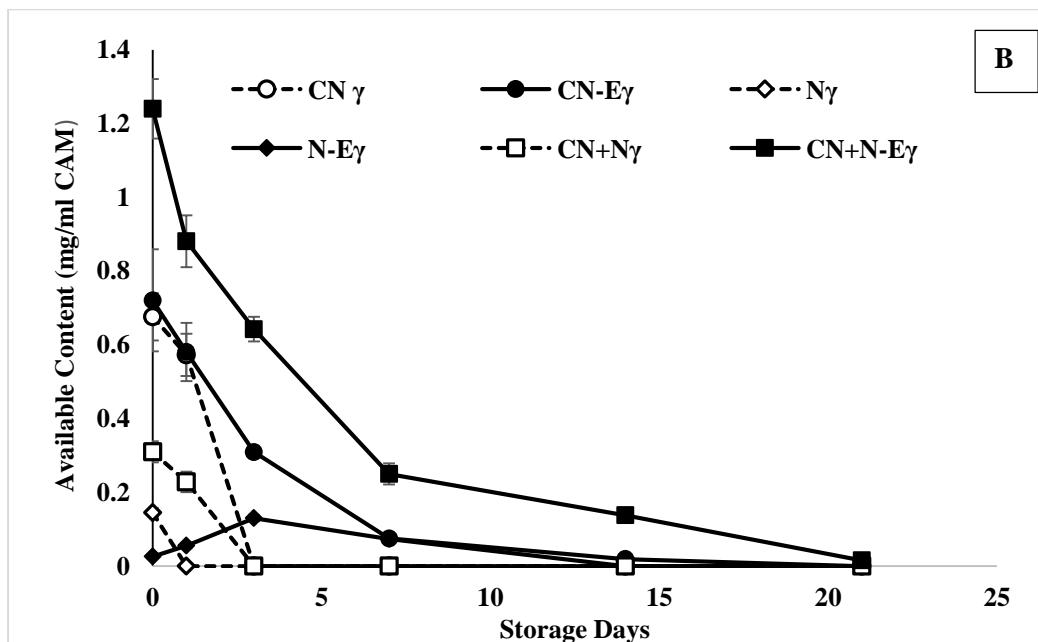
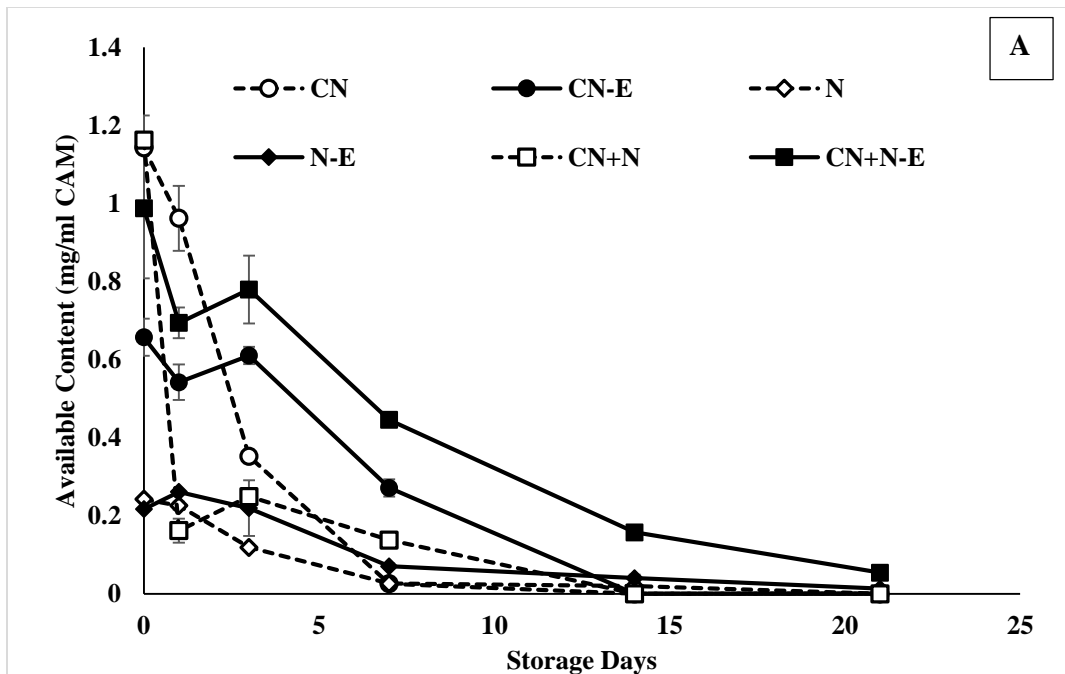


Figure 4.3: Effect of γ -irradiation on available free or microencapsulated antimicrobials, mg/ml CAM (*Cinnamomum cassia* and nisin) against *L. monocytogenes* during storage at 4° C in *in vitro* BHI-agar deep well model. A) without irradiation and B) with irradiation.

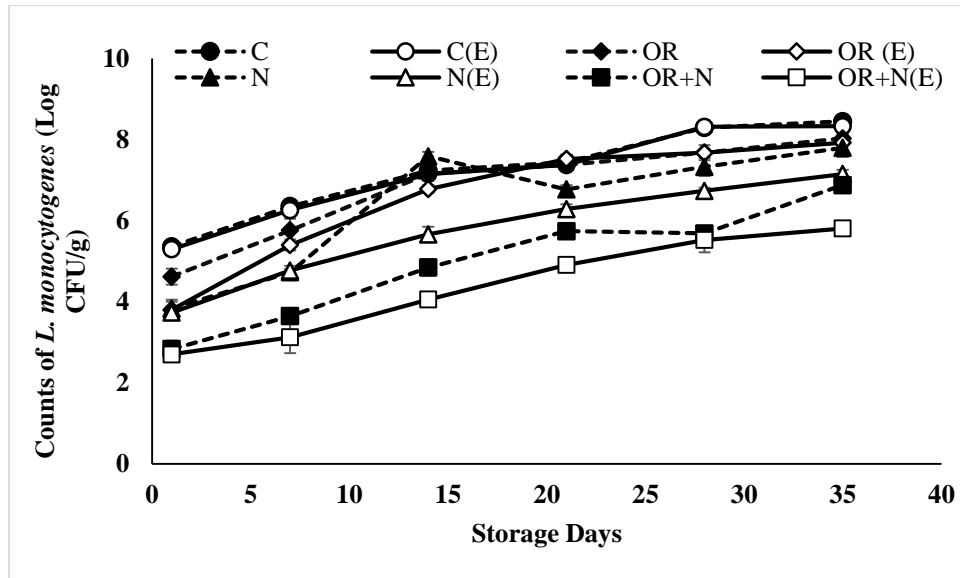


Figure 4.4: Effect of microencapsulated *Origanum compactum* + nisin on RTE cooked ham during storage at 4°C without irradiation. Dotted lines represent without microencapsulation.

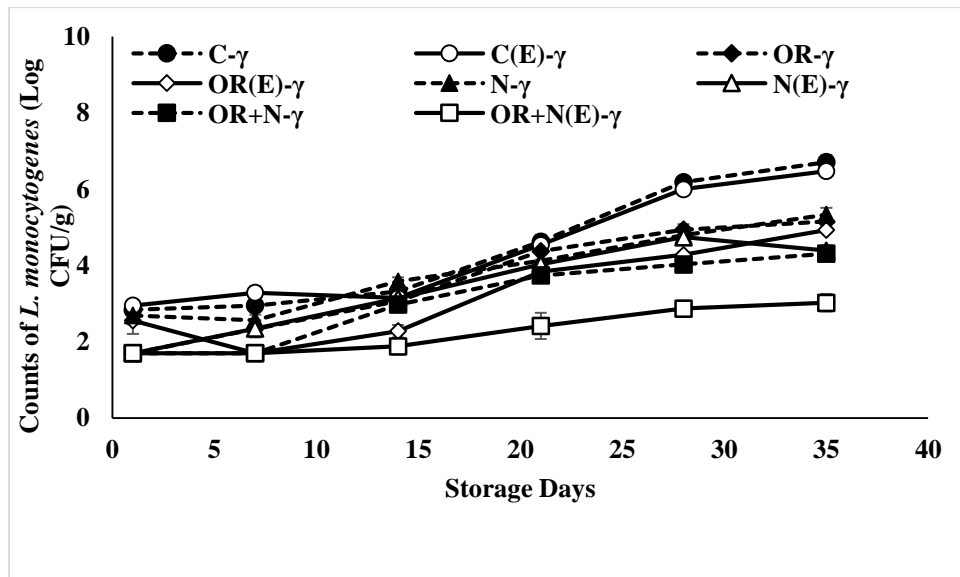


Figure 4.5: Synergistic effect of microencapsulated *Origanum compactum* + nisin and γ -irradiation on RTE cooked ham during storage at 4°C. Dotted lines represent without microencapsulation. Where, γ indicates formulation irradiated at 1.5 kGy.

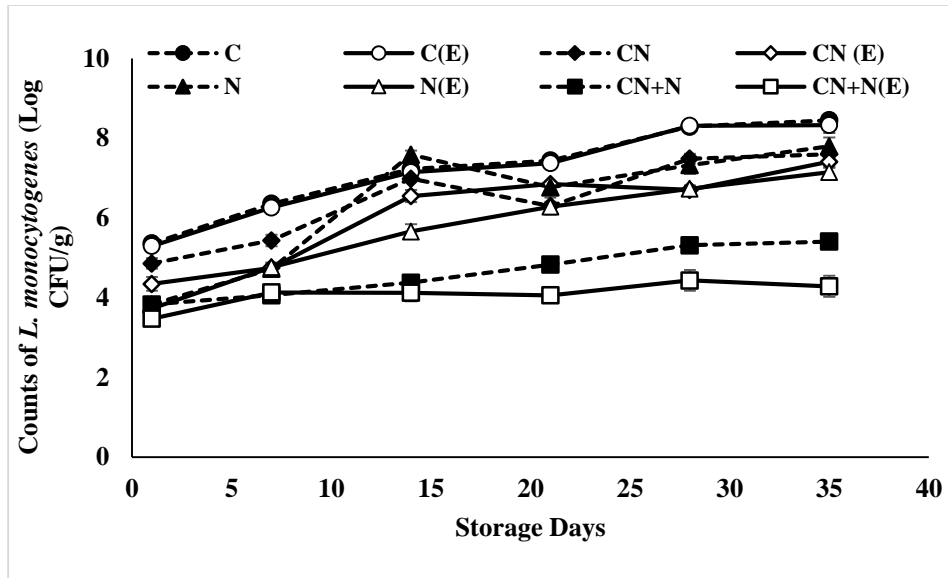


Figure 4.6: Effect of microencapsulated *Cinnamomum cassia* + nisin on RTE cooked ham during storage at 4°C without irradiation. Dotted lines represent without microencapsulation.

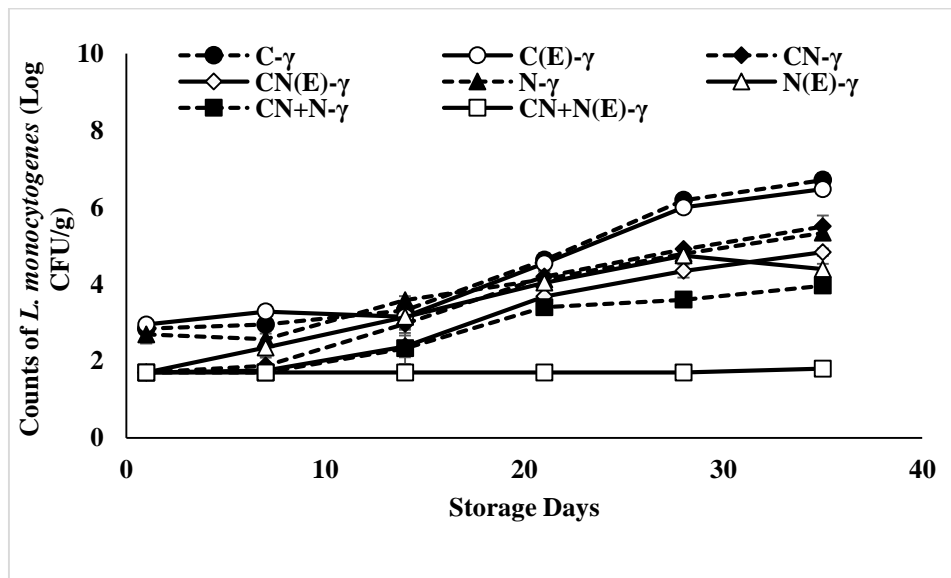


Figure 4.7: Synergistic effect of microencapsulated *Cinnamomum cassia* + nisin and γ -irradiation on RTE cooked ham during storage at 4°C. Dotted lines represent without microencapsulation. Where, γ indicates formulation irradiated at 1.5 kGy.

Table 4.1: Growth rate (ln CFU/g/day) of *L. monocytogenes* of free and microencapsulated antimicrobial compounds on RTE cooked ham.

Without γ -irradiation	Growth rate (ln CFU/g/day)	With γ -irradiation	Growth rate (ln CFU/g/day)
C	0.23	C- γ	0.29
OR	0.21	OR- γ	0.25
CN	0.18	CN- γ	0.27
N	0.22	N- γ	0.19
OR+N	0.20	OR+N- γ	0.14
CN+N	0.11	CN+N- γ	0.17
C(E)	0.24	C(E) - γ	0.26
OR(E)	0.24	OR(E) - γ	0.20
CN(E)	0.20	CN(E) - γ	0.26
N (E)	0.21	N (E) - γ	0.19
OR+N(E)	0.19	OR+N(E) - γ	0.13
CN+N (E)	0.05	CN+N (E) -γ	0.03

Table 4.2: D_{10} and radiosensitivity (RS) of *L. monocytogenes* of free and microencapsulated antimicrobial compounds on RTE cooked ham.

	D_{10} (kGy)	RS
C	0.54 ($R^2=0.99$)	1.00
OR	0.45 ($R^2=0.90$)	1.20
CN	0.29 ($R^2=0.67$)	1.86
N	0.28 ($R^2=0.96$)	1.93
OR+N	0.26 ($R^2=0.82$)	2.08
CN+N	0.23 ($R^2=0.99$)	2.35
C(E)	0.55 ($R^2=0.99$)	1
OR(E)	0.43 ($R^2=0.91$)	1.28
CN(E)	0.33 ($R^2=0.92$)	1.67
N (E)	0.30 ($R^2=0.68$)	1.83
OR+N(E)	0.19 ($R^2=0.87$)	2.89
CN+N (E)	0.11 ($R^2=1.00$)	5.00

4.11 General Discussions of the Publication-3

In this research, results showed a synergistic effect of microencapsulated antimicrobials compounds with γ -irradiation. Microencapsulated nisin and *Cinnamomum Cassia* microbeads combined with γ -irradiation showed a 35 days lag phase during storage on RTE meat products against *L. monocytogenes*. Microencapsulated nisin and *Cinnamomum Cassia* microbeads also increased the radiosensitivity of *L. monocytogenes*. Now-a-days, γ -irradiation is getting interest on preservation of RTE meat products in many countries. Microencapsulated antimicrobial microbeads in combination with γ -irradiation will open a new path for RTE meat preservation process.

Chapter-5

Patent Application

Development of Cellulose Nanocrystal (CNC) Reinforced Alginate Microcapsule to Protect the Viability of *Lactobacillus rhamnosus* ATCC 9595 During Storage and Gastric Passage

Tanzina Huq¹, Bernard Riedl², Jean Bouchard³, Carole Fraschini³ and Monique Lacroix^{1*}

¹Research Laboratories in Sciences Applied to Food, Canadian Irradiation Centre (CIC),

INRS-Institut Armand-Frappier, Université du Québec, 531 Boulevard des Prairies, Laval,
Québec, H7V 1B7, Canada

²Département des sciences du bois et de la forêt, Faculté de foresterie, géographie et géomatique,
Université Laval, Québec, G1V0A6, Canada

³FPIinnovations, 570 Boulevard St. Jean, Pointe-Claire, Québec, H9R 3J9, Canada

* **Corresponding Author:** Prof. Monique Lacroix

Telephone: +1-450-687-5010; Fax: +1-450-686-5501; **E-mail:** monique.lacroix@iaf.inrs.ca

5.1 Contributions of the Authors

All the experiments, data analysis and draft have been accomplished by Tanzina Huq with the direction of Prof. Monique Lacroix, my PhD director and co-direction of Prof. Bernard Riedl. During my experiments, Prof. Lacroix helped me to correct my protocols and also helped to solve experimental problems. Both Prof. Lacroix and Prof. Bernard Riedl have corrected the main draft and helped me to improve the discussions of the publications. Dr. Jean Bouchard and Dr. Carole Fraschini have corrected the draft.

5.2 Specific Objectives of the Patent Application

According to Food and Agriculture Association of the United Nations (FAO) and World Health Organisation (WHO), probiotics can be described as ‘live microorganisms which when administered in adequate amounts confer a health benefit on the host’. Probiotics showed a rapid loss of viability under gastric treatment and during storage at room temperature. In publication-4, the microencapsulation formulations were developed to improve the viability of probiotic. The specific objectives of this publication were to optimize the microencapsulation matrix composition during freeze drying of probiotic, evaluate the viability of probiotic under the treatment of gastrointestinal tract and during storage using the optimized formulation. Finally, microencapsulating matrix were characterized by swelling and SEM.

5.3 Résumé

Le *Lactobacillus rhamnosus* ATCC 9595 a été encapsulé dans des microbilles d'alginate -CNC - lécithine pour la production de microcapsules nutraceutiques. La concentration de CNC et de lécithine dans des microbilles d'alginate a été optimisée en contrôlant la viabilité de *L. rhamnosus*, après stabilisation des microbilles par lyophilisation. Les résultats ont montré que les microbilles d'alginate (AC-0) ont diminué la viabilité bactérienne de 0.95 log UFC /g alors que l'addition de 13 % en poids de CNC dans les microbilles d'alginate (AC-3) ont réduit la viabilité bactérienne ($P > 0.05$) par 0.01 log UFC/g après lyophilisation. L'incorporation de 2 % en poids de lécithine dans les microbilles d'alginate CNC (ACL-1) a également eu un effet protecteur pendant le séchage de même que chez les microbilles d'alginate CNC (AC-3). La résistance à la compression des microbilles ACL-1 lyophilisés a été améliorée de 40 % par rapport à microbilles d'alginate seules. Des études de gonflement ont montré que l'addition de CNC et de la lécithine dans les microbilles d'alginate ont diminué (environ 47%) l'absorption de liquide gastrique mais ont augmenté le temps de dissolution de 20 min des microbilles d'alginate (A-0). Pendant la transition complète à travers le passage gastrique, la viabilité de *L. rhamnosus* dans les microbilles ACL- 1 séchées est de 52% plus élevée par rapport à *L. rhamnosus* A-0 encapsulés dans des billes de base. La viabilité de *L. rhamnosus* a également été évaluée au cours du stockage à 25 et 4°C. Il a été constaté que, à 25 et 4°C, la viabilité de *L. rhamnosus* encapsulé dans les microbilles ACL-1 a diminué de 1.23 et de 1.08 log, respectivement, alors que l'encapsulation dans des microbilles A-0 a donné une réduction de 3.17 et de 1.93 log, respectivement.

5.4 Abstract

Lactobacillus rhamnosus ATCC 9595 cells were encapsulated in alginate-CNC-lecithin microbeads for the production of nutraceutical microcapsules. The concentration of CNC and lecithin in alginate microbeads was optimized by monitoring the viability of *L. rhamnosus* after stabilization of the microbeads by freeze-drying. Results showed that alginate microbeads (AC-0) reduced by 0.95 log CFU/g whereas addition of 13% w/w CNC in alginate microbeads (AC-3) reduced ($P > 0.05$) by 0.01 log CFU/g of bacterial viability after freeze drying. Incorporation of 2% w/w lecithin in alginate-CNC microbeads (ACL-1) also revealed a protective effect during free drying similar to alginate-CNC microbeads (AC-3). The compression strength of the freeze-dried ACL-1 microbeads improved by 40% compared to alginate microbeads alone. Swelling studies revealed that addition of CNC and lecithin in alginate microbeads decreased (around 47%) the gastric fluid absorption but increased the dissolution time by 20 min compared to alginate microbeads (A-0). During complete transition through the gastric passage, the viability of *L. rhamnosus* in dried ACL-1 microbeads was 52% higher as compared to *L. rhamnosus* encapsulated in A-0 based beads. The viability of *L. rhamnosus* was also evaluated during storage at 25 and 4°C. It was found that at 25 and 4°C storage conditions, the viability of *L. rhamnosus* encapsulated in ACL-1 microbeads decreased by 1.23 and 1.08 log, respectively, whereas the encapsulation with A-0 microbeads exhibited a 3.17 and 1.93 log reduction, respectively.

Key Words: Cellulose Nanocrystals, Probiotic, Microcapsule, Alginate, Freeze Drying, Water Activity.

5.5 Introduction

Probiotics are defined as live microbial food ingredients that have a beneficial effect on human health. Lactic acid bacteria used as probiotics are commonly incorporated into foods to provide a wide variety of health benefits (Hansen *et al.*, 2002; Chou and Weimer, 1999). The administration of probiotic bacteria in the nutraceutical sector has rapidly expanded in recent years, with a predicted global market worth \$32.6 billion by 2014. Today, this market is growing at a pace of 5 to 30% depending on the country and product type. Many of the health promoting claims attributed with probiotics are dependent on the cells being both viable and sufficiently numerous in the intestinal tract (Cook *et al.*, 2012). In order to provide health benefits from probiotic bacteria, it has been recommended that they must be present at a minimum level of 10^6 CFU/g of food product or 10^7 CFU/g at point of delivery or be eaten in sufficient amounts to yield a daily intake of 10^8 CFU/g (Chavarri, 2010; Picot and Lacroix, 2004). After ingestion, many probiotic strains showed a considerable loss of viability as they pass through the low pH of the stomach and the high bile salt conditions of the intestine (Charteris *et al.*, 1998). The important role of lactic acid bacteria as starters in the elaboration of dairy and pharmaceutical products highlights the requirement of appropriate processes for their preservation. Among the different preservation methods available, freeze-drying has been the method of choice for the storage and delivery of microbial cultures from collections. However, during this process, the number of viable bacteria is dramatically reduced following damage of the nucleic acids and enzymes of the bacterial membranes. Probiotics are also very unstable at room temperature during storage due to the protein denaturation and damage of cell wall and cell membrane (Tymczyszyn *et al.*, 2011; Chana *et al.*, 2010).

Microencapsulation is a process that is gaining interest for introducing viable probiotic in foods and nutraceuticals. It is important that the microencapsulation offers good protection during the

stabilization process (freeze-drying), during storage at room temperature and during the gastrointestinal passage. In addition, the polymer used must be non-cytotoxic, as well as non-antimicrobial to ensure that the host, as well as the bacteria, are not harmed by it (Capela *et al.*, 2006; Huq *et al.*, 2013). Alginate, the most widely used encapsulating material, is a linear polysaccharide consisting of β -(1 \rightarrow 4)-linked D-mannuronic acid (M) and α -(1 \rightarrow 4)-linked L-guluronic acid (G) residues, derived from brown algae or bacterial sources (**Figures 5.1-A and 5.1-B**). Due to the abundance of algae in water there is a large amount of alginate material present in nature. Industrial alginate production is approximately 30,000 metric tons annually. The use of alginates is favoured because of its low cost, simplicity and biocompatibility (Khan *et al.*, 2010; Pawar and Edgar, 2012). Alginates are particularly well suited to bacterial encapsulation due to their mild gelling conditions, GRAS (generally recognised as safe) status and lack of toxicity. Alginates gel upon contact with divalent ions (*e.g.* calcium chloride), forming what has been described as an “egg box structure” (**Figure 5.1-C**) between four G residues (Wahab *et al.*, 1997). Bacteria (1–3 μ m size) are well retained in the alginate gel matrix which is estimated to have a pore size of less than 17 nm. However, the gel is susceptible to disintegrate in the presence of excess monovalent ions, Ca²⁺-chelating agents and harsh chemical environments (Klein *et al.*, 1983; Krasaekoopt *et al.*, 2004). The removal of frozen water from the alginate hydrogel matrix during the freeze-drying process distorts the beads and induces poor mechanical properties which could influence the viability of the *L. rhamnosus* (Sriamornsak *et al.*, 2007). CNC could be a new promising material to overcome this weak point of alginates. In our previous study (Huq *et al.*, 2012), we have shown that CNC has a very good reinforcing property with alginates which could improve the poor mechanical properties of alginate matrix during the stabilization process (freeze-drying). CNC is a cellulose-based nanomaterial and is composed of rod-like shaped nanoparticles

and it can be referred to as nanocrystals, whiskers or nanowhiskers. CNC forms a stable chiral nematic crystalline ordered phase in aqueous suspension (Marchessault *et al.*, 1959; Revol *et al.*, 1992). These cellulose nanocrystals are featured by an average diameter of 5–10 nm and an average length of around 100 nm. CNC is a new non-toxic nanomaterial and has good potential in the food industry (Alexandrescu *et al.*, 2013, Kovacs *et al.*, 2010). The particular grade used in this study possesses a negatively charged surface area at neutral pH, which could have interact with alginate during probiotic encapsulation by CaCl_2 (Habibi *et al.*, 2010; Beck-Candanedo *et al.*, 2005). Lecithin, which is a mixture of phospholipid compounds, can provide electrostatic and steric stabilizations with alginate by protecting the core materials (Donthidi *et al.*, 2010). In the human intestine, bile salts can disrupt the bacterial membrane of the gram-positive bacteria (such as lactic acid bacteria) which reduces their survival during the transition through the intestine (Chen *et al.*, 2012). Thus, addition of lecithin with an alginate-CNC matrix was able to overcome this issue of bile by constructing microenvironments to maximize the survival of bacteria. Starch has been applied with alginate-CNC-lecithin matrix due to its wide application in nutraceutical field for probiotic encapsulation. It is found to be readily available, cheap and edible (Chana *et al.*, 2010; Sultana *et al.*, 2000).

The aim of this work was to develop alginate-CNC-lecithin microcapsules for the encapsulation and protection of the probiotic bacteria during storage as well as provide a good release into the intestine. This study was used to optimize the concentration of CNC and lecithin with alginate during drying and also evaluate the viability of microencapsulated lactic acid bacteria during storage and gastric passage. The physicochemical behaviour of the microcapsules such as swelling at different gastric pH and mechanical property was also investigated.

5.6 Materials and Methods

5.6.1 Materials

Sodium alginate (alginic acid sodium salt from brown algae, guluronic acid or glucuronic content ~ 65 - 70%; mannuronic acid content ~5 - 35%), calcium chloride (granules), pancreatin and bile salts were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Lecithin (ALCOLEC® PC 75) was purchased from American Lecithin Company (ALC, add the city and the state (Oxford, CT, USA). CNC was supplied from FPIInnovations and was produced in FPIInnovations pilot plant (Pointe-Claire, QC, Canada) from a commercial bleached softwood kraft pulp according to a procedure modified from the literature (Dong *et al.*, 1998). Starch (Hylon VII) was purchased from National Starch Food Innovation (Bridgewater, NJ, USA).

5.6.2 Strain and Culture Preparation

Lactobacillus rhamnosus was obtained from ATCC 9595 (ATCC, Manassas, VA, USA) and stored at -80° C in Lactobacilli MRS (de Man, Rogosa and Sharpe) (De Man *et al.*, 1960) broth (Difco Laboratories, Detroit, MI, USA) supplied with 15% (v/v) glycerol added. *Lactobacillus rhamnosus* ATCC 9595 were transferred twice in MRS broth at 37° C. Culture cells were harvested after 24 h by centrifugation (4500g, 10 min at 4° C), washed and resuspended in 0.1% (w/v) peptone water to obtain a final concentration of $\sim 10^9$ - 10^{10} colony-forming units CFU/mL.

5.6.3 Microencapsulation Procedure

Sodium alginate, CNC, starch and lecithin were sterilized by γ -irradiation at a dose of 1 kGy using a ^{60}Co -source irradiator (Nordion Inc., Kanata, ON, Canada). Alginate and CNC blends were dissolved in water according to various wt% ratios (Alginate:CNC, wt% - 95:5; 91:9; 87:13; 83:17; 80:20). Then, the suspension was stirred for 24 h. Lecithin (2, 13 and 22 wt%) was mixed with the optimized CNC-alginate suspension and starch (22, 36 and 53 wt%) was mixed with the optimized

alginate-CNC-lecithin suspension by heating at 60-70°C for 30 minutes. Then the whole matrix was homogenized by using an Ultra-Turrax TP18/1059 homogenizer (Janke and Kunkel, Staufen, Germany) at room temperature and 24000 rpm for 1 min. The optimized formulations are presented in **Table 5.1**.

A volume of 12.5 mL of culture (bacterial concentration of $\sim 10^9 - 10^{10}$ CFU/mL) was gently mixed in 50 mL of bead-forming suspension. The beads were produced by releasing from a 10 mL plastic syringe through a 20-gauge needle (0.9 mm in diameter) into a beaker containing CaCl₂ solution (1% w/v) under gentle stirring at room temperature. The formed beads were then allowed to harden for 30 min and then rinsed with sterilized distilled water. The whole experimental procedure was outlined in **Figure 5.2**.

5.6.4 Freeze-drying

The microencapsulated *L. rhamnosus* beads and free cells were frozen at -80 °C for 2 to 3 h. Then the samples were freeze-dried (Labconco Freeze Dry Systems, USA) at -49°C and 0.09 torr pressure for 48 h.

5.6.5 Determination of Mechanical Strength

The mechanical strength of single freeze-dried beads was qualitatively determined using a universal tensile machine (Model H5KT, Tinius-Olsen Inc., Horsham, PA, USA,) equipped with a 100 N load cell (type FBB) and a compression probe. Single lyophilized beads were positioned on a flat platform and compressed in a vertical direction using a flat-tip probe, which was connected to a force transducer. The forces imposed on the beads and the displacement was recorded during compression.

5.6.6 Swelling and Dissolution under Simulated Gastrointestinal Conditions

Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) were prepared according to US Pharmacopeia (1995) as described before. The pH of SGF was adjusted to 2 and 5 with 1 M HCl, a suitable pH range for mimicking the conditions in the non-fasted stomach (Cook et al., 2011) and the pH of SIF was adjusted to 7.0. Unloaded microcapsules (20 mg) were placed in a vial containing simulated gastric or intestinal juice (5 mL) and placed in a water bath (37 °C, with shaking, 100 rpm). The weight of the microcapsules was measured every 30 min for 180 min for SGF and every 10 min until dissolution for SIF. The swelling (%) was calculated with respect to the dry weight using the following equation,

$$\text{Swelling (\%)} = (W_t - W_0)/W_0 \times 100 \quad (1)$$

Where W_t is the weight of the microcapsules at time t and W_0 is the weight at time 0.

5.6.7 Water Activity (a_w) and Water Content Measurement

Water activity (a_w) was measured during storage at 25 and 4° C after drying the samples using a Rotronic water activity instrument (Rotronic A_w Quick, Bassersdorf, Switzerland). The residual water content of the dried probiotic free cell powder and microbeads were determined in a drying oven at 105° C until a constant weight was attained (Tymczynszyn *et al.*, 2011).

5.6.8 Storage of Dried Microencapsulated Probiotic Beads

After freeze-drying, the microencapsulated cells and free cells were stored at 4 and 25° C. The viability of the probiotic cells was determined throughout the storage after certain time intervals (Selmer-Olsen *et al.*, 1999).

5.6.9 Determination of Survival and Inactivation Rate Constant

The stability of probiotic during storage was determined as logarithmic value of the ratio of cell count at the beginning of the storage (N_0) and at a particular storage time (N_t). To obtain the rate of inactivation during storage, the regression line was determined from the plot of the common logarithmic value of the residual bacterial count (N_t/N_0) versus the storage period (t , day) for each temperature. Each regression line fitted the following equation:

$$\log N_t / N_0 = -kt \quad (2)$$

The inactivation rate constant k (day^{-1}) was determined from the absolute value of the slope of each storage condition (Ananta *et al.*, 2005).

5.6.10 Survival of Free and Microencapsulated Probiotic in Simulated Gastric Fluid (SGF)

The SGF was formulated according to United States Pharmacopeia (1995) and composed of 3.2 g/L of pepsin (approx. 600 units/mg, with one enzyme unit producing a ΔA_{280} of 0.001/min at pH 2.0 and 37 °C, measured as tri-chloroacetic acid-soluble products from haemoglobin as a substrate) and 2.0 g/L NaCl and pH finally adjusted to 1.5 using HCl (1.0 M) solution. SGF was sterilized by filtration with a Bottle Top Vacuum Filter (0.2 μm pore size; Nalge Nunc International, NY, USA). Amounts of 1g microencapsulated probiotic beads (fresh and dried) or a volume of 1 mL of free cells were added to 10 mL of SGF for 2 h at 37° C under mild agitation (100 rpm) in a G24 Environmental Incubator Shaker (New Brunswick Scientific Co., Enfield, CT, USA). After incubation in simulated gastric solution (SGF), beads were collected and dissolved in 10 mL of sterile phosphate buffer (0.5 M, pH 7.5). For the free bacterial cells in SGF, a volume of 1.0 mL was taken and mixed in the same phosphate buffer solution. A similar procedure was applied to beaded or free cells, without SGF treatment, for the determination of initial bacterial number.

5.6.11 Survival of Free and Microencapsulated Probiotic after Sequential Incubation in SGF and SIF

The SGF and SIF were prepared according to the United States Pharmacopeia (1995). The SIF was prepared by dissolving 6.8 g of KH_2PO_4 (Laboratoire MAT) in 250 mL of water. Then, 77 mL of NaOH (0.2 N) and 500 mL of water, 1.25 g of pancreatin and 3 g of bile salts were added to the solution. Finally, the pH was adjusted at 7.0 with NaOH (0.2 N) or HCl (0.2 N). The SIF was completed with water to obtain 1000 mL. The SIF was sterilized by filtration with a Bottle Top Vacuum Filter (0.2 μm pore size). The sequential incubation analysis was based on the method described by (Rao *et al.*, 1989). Microencapsulated fresh or dried beads (1 g) were placed in a tube containing 9 mL of sterile SGF (pH 1.5) and incubated at 37° C for 2 h. After incubation, the beads were removed and placed in 9 mL of sterile SIF (pH 7.0). The tubes were then again incubated at 37° C for 180 min (3 h). After incubation, a 1.0 mL aliquot of dissolved beads was removed and analyzed.

5.6.12 Microbiological Analysis

The freshly prepared or dried microencapsulated probiotic and free cell suspension or dried powder were dissolved in 10 mL of phosphate buffer solution (pH 7.0, 0.5M) by using a Lab-blender 400 stomacher (Seward Medical, London, UK) at 260 rpm for 1 min. The plate count method was used to determine the number of total viable bacterial cells. From this homogenate, appropriate serial dilutions were prepared in 0.1% peptone, and 100 μL of each dilution was spread on MRS agar plate (Lactobacilli MRS Broth, EMD Chemicals Inc., Germany), which were incubated for 48 h at 37°C under anaerobic conditions using an anaerobic gas pack and anaerobic jars (Anaerogen, Oxoid Limited, Hampshire, England). Bacterial counts were expressed as log CFU/g.

5.6.13 Scanning Electron Microscopy (SEM) Analysis

The SEM investigation of the freeze dried bead samples was performed on an Environmental SEM (ESEM, Quanta 200 FEG, FEI Company Hillsboro, OR, USA) under high vacuum mode with an accelerating voltage of 20.0 kV and at 0°C temperature. ESEM eliminated the need for any conducting coating thus preventing any potential damage of the samples due to coating. The microscope was equipped with an energy dispersive X-ray (EDX) spectrometer (Genesis 2000, XMS System 60 with a Sapphire Si/Li detector from EDAX Inc. (Mahwah, NJ, USA).

5.6.14 Statistical Analysis

To validate the results obtained during different experimental procedure, each analysis was carried out in triplicate in a completely randomized experimental design. An analysis of variance (ANOVA) and multiple comparison tests of Duncan's were used to compare all the results. Differences between means were considered significant when the confidence interval was smaller than 5% ($P \leq 0.05$). The analysis was performed by the PASW Statistics 18 software (IBM Corporation, Somers, NY, USA). The enumeration of probiotic population was independently replicated three times ($n = 3$), with two measurements per replicate.

5.7 Results and Discussion

5.7.1 Optimization of CNC, Lecithin and Starch Concentration by Checking the Viability of the Probiotic during FreezeDrying

5.7.1.1 CNC Concentration Optimization in Alginate Microbeads

The effect of CNC (% w/w) in alginate beads (A-0) on the viability of *L. rhamnosus* is presented in **Figure 5.3(A)**. Before drying, the viability of free *L. rhamnosus* was 9.81 log CFU/g and after freeze-drying, the viability was 9.09 log CFU/g. The viability was reduced by 0.72 log during drying. Microencapsulation with alginate only could not protect the viability of probiotic during freeze-drying. The results showed that, before and after freeze-drying, the viability in alginate microcapsule (A-0) was 9.23 and 8.23 log CFU/g, respectively, which was reduced by 1 log during freeze-drying. Incorporation of 5 and 10 % w/w CNC in alginate bead (AC-1 and AC-2) showed 1.13 and 0.67 log reduction after freeze-drying. But addition of 13, 17 and 20 % w/w CNC in alginate bead (AC-3, AC-4 and AC-5) significantly ($P \leq 0.05$) improved the viability during freeze-drying, compared to AC-0, AC-1 and AC-2. The viability was found to be 9.57, 9.68 and 9.68 log CFU/g, after freeze-drying of AC-3, AC-4 and AC-5 samples respectively. Thus, addition of 13 % w/w CNC in alginate microbeads (AC-3) has permitted a complete protection of the bacterial viability during freeze-drying treatment that corresponded to the optimized concentration for *L. rhamnosus*.

5.7.1.2 Lecithin Concentration Optimization in alginate-CNC microbeads

The effect of lecithin (% w/w) in optimized alginate-CNC microbeads (AC-3) on the viability of *L. rhamnosus* during freeze drying is shown in **Figure 5.3 (B)**. After freeze drying, incorporation of 2, 13 and 22 % w/w of lecithin in alginate-CNC microbeads (ACL-1, ACL-2 and ACL-3) gave a viability 9.65, 9.55 and 9.29 log CFU/g, respectively, which was not significantly ($P > 0.05$)

different from values before freeze-drying (9.68, 9.61 and 9.29 log CFU/g, respectively). However after drying, incorporation of 2 % w/w lecithin in alginate-CNC microbeads (ACL-1) showed slightly improved viability ($P>0.05$) compared to alginate-CNC microbeads (AC-3). Thus, ACL-1 formulation was considered as an optimal concentration.

5.7.1.3 Starch Concentration Optimization in Alginate-CNC-Lecithin Microbeads

The effect of incorporation of starch (% w/w) in optimized alginate-CNC-lecithin beads (ACL-1) on the viability of *L. rhamnosus* during freeze-drying is shown in **Figure 5.3 (C)**. Before freeze-drying, the addition of 22, 36 and 53 % w/w of starch in alginate-CNC-lecithin microbead (ACLS-1, ACLS-2 and ACLS-3) resulted in a bacterial viability 9.03, 9.48 and 9.43 log CFU/g, respectively, and after freeze-drying, viability values were 8.61, 9.12 and 9.03 log CFU/g, respectively. Results showed that addition of 36 % w/w starch in alginate-CNC-lecithin microbeads (ACLS-2) significantly ($P\leq 0.05$) increased the viability by 14% as compared to the addition of 22 % w/w of starch in alginate-CNC-lecithin microbeads (ACLS-1). The increase of starch concentration did not improve the viability of *L. rhamnosus*. Thus 36 % w/w of starch (ACLS-2) was found to be the optimal concentration, in agreement with the results of Chana *et al.* (2010).

Freeze-drying is a widely used dehydration method for cell preservation. There have been a number of reports regarding the adverse effects of freeze-drying on cellular structure and cell functions, which can eventually lead to the loss of cell viability. Among the effects reported were protein denaturation, damage to the cell wall, RNA/DNA, cell membrane and also unfavorable oxidation reactions (Chan and Zhang, 2002). It had been reported that the Ca–alginate hydrogel matrix alone could not protect the encapsulated cells (Champagne *et al.*, 1992; De Giulio *et al.*, 2005). This finding is in good agreement with the present work. Moreover, alginate has a very

highly porous structure and that is why it has more surface area in which the cells could be directly exposed to freeze-drying. This exposure could decrease cell viability since it is reported that the probability of cell death during freeze-drying is proportional to the exposed surface area (Bozoglu *et al.*, 1987). This difficulty could be overcome by addition of filler compounds that have a reinforcing effect on the biopolymer matrix. Chana *et al.* (2010) reported that the incorporation of filler could affect the viability of microencapsulated cells in Ca–alginate beads during freeze-drying. In this experiment, we observed that 13 % w/w CNC (AC-3) with alginate matrix improved the viability of lactic acid bacteria after freeze drying. According to our previous study (Huq *et al.*, 2012), we showed that CNC improved the physico-chemical properties of alginate based matrix and the optimized value was 5 % w/w CNC with 95 % w/w alginate matrix. We found that addition of 5 % w/w CNC in alginate matrix improved the mechanical property by 37% and also reduced the swelling by 53% compared to control alginate matrix. To verify the stability of probiotic at storage and gastric passage, we kept both 5 and 13 % w/w CNC with 95 and 87 % w/w alginate formulations, respectively, for comparison.

In present study, lecithin also played an important role for improving the viability of lactic acid bacteria during freeze-drying. Chen *et al.* (2012) reported that lecithin could be an interesting vehicle when used with alginate and chitosan for encapsulation of probiotics. But an excess of lecithin has a detrimental effect on the alginate matrix during freeze drying. Because lecithin is a phospholipid fatty compound, it could increase the moisture content inside the alginate-CNC matrix (Arcolego *et al.*, 1997). From our study, 2 % w/w lecithin with alginate-CNC (ACL-1) did not show any detrimental effect on the viability of the probiotic during freeze-drying. Starch was also used in this study to verify the viability of probiotic but the formulation containing 36 % w/w

starch (ACLS-2) was not able to improve the viability of *L. rhamnosus* compared to the formulation made with 13 % w/w CNC and 2 % w/w lecithin.

5.7.2 Mechanical Property of the Freeze Dried Beads

Figure 5.4A represents the effect of CNC in an alginate matrix on compression strength. The compression strength was 43, 58, 54, 56, 54 and 57 MPa after incorporation of 0, 5, 9, 13, 17 and 20 % w/w of CNC into the alginate microbeads, respectively. These results showed that the incorporation of 5 and 13 % w/w CNC into an alginate bead matrix improved the mechanical property by 35 and 30% ($P \leq 0.05$), respectively, compared to the freeze dried alginate beads alone. Incorporation of more than 13 wt% CNC into alginate bead matrix did not show an improvement of mechanical property ($P > 0.05$).

The compression strength of all optimized formulations (A-0, AC-1, AC-3, ACL-1 and ACLS-2) is shown in Figure-5.4 B. It was found that formulations AC-1, AC-3 and ACL-1 showed 58, 56 and 60 MPa compression strength, respectively. The addition of 13 % w/w CNC and 2 % w/w lecithin improved the mechanical property of the freeze-dried alginate ($P \leq 0.05$). But addition of 36 % w/w starch (ACLS-2) gave a compression strength value of 41 MPa which is close to alginate compression strength (43 MPa). Thus, the ACL-1 formulation improved the compression strength by 40% as compared to alginate microcapsule (A-0) alone.

The quality of freeze-dried alginate microcapsules can be either spongy and not friable or fragile. The reason for the variation in the physical characteristics is not clear, but it could be due to the freeze-drying conditions or the amount of residual water within the microcapsule after the freeze drying process. If the microcapsules are fragile and friable, they can cause problems during handling, storage and further processing since they can be easily broken and disintegrated. The addition of CNC (as reinforcing filler) and lecithin were found to minimize these problems, as the

microcapsules were rigid and not friable. These characteristics are due to the filler reinforcing in the hydrogel network and filling the voids (Chana *et al.*, 2010; Tal *et al.*, 1999). The improvement of compression strength is attributed to a good interfacial interaction between CNC and alginate bead matrix because of similar polysaccharide structures of cellulose and alginate (Chang *et al.*, 2010) and also it is believed that CNC could reduce the porosity of the alginate matrix during freeze-drying. These results showed a resemblance with the protection of the viability of *L. rhamnosus*. But incorporation of starch (more filler) makes the microcapsule more brittle which can crack at a lower applied force and lead to fewer voids and denser packing. As a result, the beads lost their flexibility and became brittle.

5.7.3 Swelling and Dissolution under Simulated Gastrointestinal Conditions

The main objective of this experiment was also to evaluate the behavior of the microcapsules during their transition through the stomach and intestine. Swelling behaviour of freeze-dried beads indicates the speed and easiness of a liquid to penetrate the alginate–CNC–lecithin matrix, as a necessary step for lactic acid bacteria release. The swelling behaviour of the microcapsules (A-0, AC-1, AC-3, ACL-1 and ACLS-2) at pH 1.5 and 5 are represented in **Figure 5.5**. The results are plotted as the percentage of swelling (%) versus time. The swelling of the microcapsules for all formulations showed similar trends at both pH (1.5 and 5). At pH 1.5, the swelling of the alginate microcapsules (A-0) was 377 % whereas the swelling of AC-3, ACL-1 and ACLS-2 formulations was around 200 % after 120 min transition. Incorporation of CNC or lecithin or starch significantly ($P \leq 0.05$) decreased the absorption of gastric fluid compared to alginate, which represents less destruction of bacterial cell. At pH 5, the microcapsules showed a similar swelling behaviour. These results demonstrated that formulation AC-3, ACL-1 and ACLS-2 would have a protective effect, until reaching the small intestine, compared to control alginate microcapsules. Chan *et*

al.(2009) reported that at low pH, alginic acids are protonated, resulting in an uncharged polymer with limited solubility leading to rapid polymer rearrangement, expelling water out from the gel network and contracting to the original relaxed gel volume, though alginate shows a greater swelling due to its inside porous structure. On the other hand, incorporation of CNC is thought to increase the tortuosity in the alginate bead matrix leading to slower diffusion processes. The swelling properties are enhanced if the filler is less permeable than the matrix and has a good dispersion within the matrix (Huq *et al.*, 2012; Sanchez-Garcia *et al.*, 2010). Our results indicated that after incorporation of 13 % w/w CNC (AC-3) swelling decreased by 47% compared to alginate (A-0). Thus it has also been demonstrated that 13 % w/w of CNC has an impact to reduce the porous structure of alginate.

The swelling of the dried microcapsules in simulated intestinal fluid is shown in **Figure-5.5**. When the microcapsules are placed in the simulated intestinal fluid of pH 7, the Na⁺ ions present in the external solution undergo an ion-exchange process with Ca²⁺ ions which can bind with -COO⁻ groups, mainly in the polymannuronate sequences. As a result, the electrostatic repulsion among negatively charged -COO⁻ groups increases which ultimately causes chain relaxation and enhances the gel swelling. Thus it can be said that in the initial phase of the swelling process the Ca²⁺ ions present in polymannuronate units are exchanged with Na⁺ ions, thus causing the beads to swell along with absorption of water. Therefore, phosphate buffer is mainly responsible for the swelling of the microcapsules. The Na⁺ ions undergo ion exchange with Ca²⁺ ions thus making the bead structure looser, and phosphate ions interact with calcium ions to form calcium phosphate. In this way, the swelling and water absorption of the beads seem to be due to the presence of sodium phosphate buffer (Bajpai and Sharma, 2004). On the other hand, the carboxylic acid moieties are deprotonated as the pK_a values of glucuronic and mannuronic monomers are 3.65, and 3.38,

respectively. This increases polymer hydrophilicity and sets up an electrostatic repulsion, which subsequently expands the gel network, leading to a swollen gel state (Cook *et al.*, 2011; Almeida and Almeida, 2004; Chan *et al.*, 2009).

Because of very high swelling behaviour and therefore high susceptibility to mechanical damage, large errors are associated with each measurement (even after incorporation of CNC and lecithin). This confirms that the microcapsules should start to dissolve upon entry in the small intestine. According to the results, it was observed that alginate (A-0) and alginate with 5 % w/w of CNC (AC-1) showed complete dissolution at after 40 min whereas incorporation of 13 wt% CNC and/or 2 % w/w lecithin and/or 36 % w/w starch (AC-3, ACL-1 and ACLS-2, respectively) showed complete dissolution at after 60 min transition through the intestinal fluid. These results demonstrated that incorporation of CNC or lecithin or starch increased the dissolution time at which microcapsules can maintain the target release of the lactic acid bacteria. Cook *et al.* (2011) showed a similar improvement of dissolution time for alginate microcapsule in intestinal fluid with chitosan coating.

5.7.4 Storage of Dried Free and Microencapsulated *L. rhamnosus*

In order to verify the bacterial resistance during storage, the encapsulated lactic acid bacteria were stored at 4 and 25°C up to 42 days. In our present study, the mean residual water content of all types of samples after drying was found to be between 3-4%, with no significant difference between free cell and encapsulating matrix. The viabilities of microencapsulated *L. rhamnosus* in different formulations during storage (at 25 and 4 °C) are presented in **Figure 5.6A and 6B**.

Results showed that at 25°C (**Figure 5.6A**), free cell, A-0 and AC-1 exhibited a rapid degradation up to 42 days of storage and it was found that the viability was reduced by 4.36, 3.17 and 3.16 log, respectively. Results obtained for microencapsulated *L. rhamnosus* in AC-3, ACL-1 and ACLS-2

showed less degradation of the viability during storage at 25°C. The viability in AC-3, ACL-1 and ACLS-2 was reduced by 1.73, 1.23 and 2.66 log respectively, after 42 days of storage. These studies revealed that AC-3 and ACL-1 improved the storage viability by 45 and 61%, respectively compare to A-0. Donthidi *et al.* (2010) reported that lecithin contained in microcapsules protected the viability of lactic acid bacteria above 6 log CFU/g up to 6 weeks of storage at 23°C which are similar results with our experiment while Crittenden *et al.* (2006) stated that free cells could not survive at 25°C after 4 weeks of storage.

At 4°C storage conditions (**Figure 5.6B**), AC-3 and ACL-1 also showed better protection of *L. rhamnosus* up to 42 days. It was found that AC-3 and ACL-1 showed respective viabilities from 9.57 to 8.32 and from 9.65 to 8.57 log CFU/g which was reduced by 1.25 and 1.08 log after 42 days of storage, respectively. On the other hand, the viability of *L. rhamnosus* in alginate microbeads was reduced by 1.93 log after 42 days of storage. Therefore it was revealed that AC-3 and ACL-1 improved the viability by 35 and 44% as compared to A-0 at 4°C storage conditions. Thus, AC-3 and ACL-1 displayed the highest protection of *L. rhamnosus* at both storage temperatures (25 and 4 °C) whereas free cell, formulation A-0 and AC-1 showed lower protection.

From the stability test data under two different storage conditions (25 and 4°C), we observed that the stability of *L. rhamnosus* could well be described as a first-order kinetic reaction, as indicated by Equation 2, which is supported by previous reports (Abe *et al.*, 2009; Higl *et al.*, 2007). **Table 5.2** represents the inactivation rate constant of *L. rhamnosus* during storage at 25 and 4°C. In our study, the inactivation rate at 4° C for all formulations was less than observed at 25°C storage conditions. It is recommended that free cell and microencapsulated probiotics must be stored at 4° C (Gardiner *et al.*, 2000). In order to get the highest viability, cold storage is advantageous for prolonged stabilization of probiotic. A temperature close to above 0° C generally leads to higher

viability compared to more elevated storage temperatures, because lower temperatures result in reduced rates of detrimental chemical reactions, such as fatty acid oxidation (Higl *et al.*, 2007). Also, it has been speculated that the presence of oxygen can lead to oxidation reactions that cause protein denaturation and phospholipid degradation of dried probiotic cells (Castro *et al.*, 1995). Heidebach *et al.* (2010) reported that free cell and microencapsulated probiotics showed better stability at 4°C than 25°C storage conditions. Our study also revealed a similar stability for free cell and microencapsulated *L. rhamnosus* formulations. Results showed that the addition of 13 % w/w CNC and 2 % w/w lecithin in alginate microbeads (ACL-1) exhibited an inactivation rate of 0.03 at 25 °C and 0.02 at 4°C.

It has to be noted that not only storage temperature but also water activity (a_w) plays an important role on the protection of probiotics during storage. The a_w -values at 25 and 4°C are shown in **Table 5.3A and 3B**. Results showed that at both temperatures, initially free cell showed the lowest a_w -value (0.05) compared to the micro encapsulated samples but after 42 days of storage, the a_w -value of free cell was 0.29 and 0.18 at 25 and 4 °C, respectively. During storage at 25 and 4°C, alginate microbeads (A-0) also showed high a_w -values (0.33 and 0.24, respectively). However, ACL-1 showed a_w -value of 0.12 at 25°C and 0.09 at 4°C storage, respectively. Foerst *et al.* (2012) reported that freeze dried *L. paracasei* with a_w -value of 0.33 exhibited high cell inactivation but at an a_w -value of 0.07 showed lower cell inactivation during storage at 20°C. During storage, oxygen plays an important role on the viability of dried bacteria, leading to detrimental oxidative changes on the cell membrane. It was suggested that in dried hydrogel-microcapsules the diffusion of oxygen can be limited during storage where the free cell is directly exposed to oxygen (Kurtmann *et al.*, 2009; Castro *et al.*, 1995; Selmer-Olsen *et al.*, 1999). From this point, microencapsulation of *L.*

rhamnosus with CNC and lecithin in alginate microbeads (ACL-1) could be an important matrix to protect the viability of probiotics during storage.

5.7.5 Survival of Free and Microencapsulated L. rhamnosus in SGF

The survival of free and microencapsulated *L. rhamnosus* (for wet and dry microcapsules) in SGF (2 h) treatment are shown in **Table 5.4**. The log viability was checked after 2 h treatment in SGF in order to demonstrate how much of the probiotic could reach to intestine.

After ingestion, a microencapsulated probiotic passes quickly through the oesophagus (taking around 10–14 s) and reaches the stomach which is the point at which the greatest loss of viability is expected due to high levels of acid (Cook *et al.*, 2012). In this study, the SGF test was performed for 2 h since the half gastric emptying time has been estimated using ¹³C breath tests to be around 80.5 min (Hellmig *et al.*, 2006). The emptying of the stomach contents is usually a result of peristaltic action known as the migrating myoelectric complex, whilst the pyloric sphincter ensures retention of large, insufficiently digested contents. Therefore, smaller particles will be retained for a shorter length of time in stomach (Janssens *et al.*, 1983).

L. rhamnosus (free cell) was shown to have a viability below the detection label even after short exposure (30 min) to SGF at pH 1.5 (data showed after 2 h exposure). Free *L. rhamnosus* is very sensitive at lower pH as described by Le-Tien *et al.* (2004) but microencapsulation protected the bacteria during gastric transition. From **Table 5.4**, formulations A-0, AC-1, AC-3, ACL-1 and ACLS-2 (before freeze-drying) reduced the viability by 1.45, 1.56, 0.85, 0.82 and 1.04 log, respectively, after 2 h SGF treatment. It was found that prior to freeze drying the AC-3 and ACL-1 formulations improved the viability by 41 and 43% as compared to alginate microbeads alone. Similarly, after freeze-drying A-0, AC-1 and ACLS-2 reduced the viability by 1.49, 1.57 and 1.56 log, respectively, after 2 h SGF treatment. Cook *et al.* (2011) reported that the bacterial viability

was reduced by 2.8 log CFU/g even after coating treatment of alginate with chitosan (dry microcapsule) whereas our formulations AC-3 and ACL-1 (dry), viabilities were reduced by only 0.93 and 0.94 log CFU/g. Thus, the AC-3 and ACL-1 formulations increased the viability by 38 and 37% compared to dried alginate microbeads. This study demonstrates that the incorporation of 13 % w/w CNC and 2 % w/w lecithin in alginate microbeads (both wet and dry conditions) enhanced the probiotic survival in a gastric environment.

Other researchers (Cook *et al.* 2011, Chavarri *et al.*, 2010, Krasaekoopt *et al.*, 2004, Lee *et al.*, 2004, Mandal *et al.*, 2006, and Truelstrup Hansen *et al.*, 2002) showed that microencapsulation of probiotics in alginate could not protect the bacteria in SGF. Alginate gel has a porous structure which is generally 5-200 nm in width (Gombotz and Wee, 1998). The gastric fluid (pH 1.5) can penetrate these pores and reduce the viability of probiotics. Because this gastric fluid, which in the stomach contains pepsin, a proteolytic enzyme which could break down proteins of the bacterial membrane (Cook *et al.*, 2012). In our study, it was observed that CNC and lecithin with alginate could improve the protection of probiotics in SGF. This study demonstrated that CNC and lecithin could reduce the porous structure of alginate by preventing the diffusion of acid into the alginate microcapsule and enhance protection of probiotics against SGF. But in the current study, CNC acted as a better filler than starch with alginate. This could be due to the nanocrystalline property and the nanosize of CNC, which is very suitable to reduce the porous structure of alginate molecules. Whereas, starch can be easily aggregated, resulting in a bigger alginate microcapsule size. It was also reported by Sultana *et al.* (2000), that alginate-starch microspheres (in the size range of 1.0 mm) did not improve the viability of probiotics after exposure to acidic solutions.

Our results suggested that free *L. rhamnosus* is sensitive to the gastric acidic environment and that ingestion of unprotected lactic acid bacteria would result in reduced viability. Owing to this,

microencapsulation of *L. rhamnosus* was performed and it was found that alginate alone is not enough to keep the bacteria at proper levels ($10^6 - 10^7$ CFU/g) but incorporation of CNC and lecithin can ensure greater survival in the gastric environment (more than 8 log CFU/g after SGF treatment).

5.7.6 Survival of Free and Microencapsulated Probiotics after Sequential Incubation from SGF to SIF

During passage through the gastrointestinal tract, the microcapsules should be first exposed to the low pH of the stomach and then at higher pH values as they move into the small to large intestine. The aim of this part of the work was to study how the bacteria were expelled from the microcapsules to understand in which regions of the gut the bacteria are likely to be deposited. For lactic acid bacteria to exert positive health effects, they have to colonize into the colon in large quantities (Sultana *et al.*, 2000). Microencapsulation might increase colonization by the lactic acid probiotic at the intestine because the biopolymers have muco-adhesive properties to the mucosal membranes lining of the epithelial tissues. The exact mechanism for muco-adhesion is not fully understood, but is believed to be based on the interpenetration of the muco-adhesive macromolecules and mucus, followed by the establishment of several types of interactions, including electrostatic, hydrogen bonding and hydrophobic interactions (Khutoryanskiy, 2011).

The release profile from SGF (2 h) to SIF (3 h) of free and microencapsulated *L. rhamnosus* (for wet and dry microcapsules) is shown in **Figure 5.7A and 7B**. Our previous SGF treatment study demonstrated that AC-3 and ACL-1 formulations could protect the probiotics during gastric passage which revealed a good relation with the sequential transition (from SGF to SIF) study. Alginate (A-0) microcapsules reduced the viability from 9.21 to 6.45 log CFU/g (2.75 log reduction in wet microbeads) and from 8.27 to 5.36 log CFU/g (2.91 log reduction in dry beads)

after complete transition through SGF to SIF. Lee *et al.* (2004) also reported that the bacterial population in alginate microcapsules was 1.96 log CFU/g after complete transition from SGF to SIF. The authors used chitosan coatings on alginate to improve the viability of probiotics but the viability was found only 3.18 log CFU/g after sequential transition through SGF to SIF. However, our study revealed that incorporation of 13 % w/w CNC (AC-3) in alginate microcapsule significantly ($P \leq 0.05$) improved the viability from 9.58 to 7.51 log CFU/g (2.07 log reduction in wet microbeads) and 9.57 to 7.67 log CFU/g (1.90 log reduction for the dry microbeads). Thus, the addition of 13 % w/w CNC in alginate microbeads increased the viability by 31% under wet conditions and by 30% under dry conditions as compared to alginate (A-0) alone after 2 h SGF + 3 h SIF treatment. On the other hand, addition of 2 % w/w lecithin (ACL-1) in alginate-CNC microbeads gave viabilities from 9.68 to 8.68 log CFU/g (1 log reduction in wet microbeads) and 9.65 to 8.26 log CFU/g (1.39 log reduction in dry microbeads) after complete transition. Thus ACL-1 formulation improved the viability by 63% under wet conditions and by 52% under dry conditions as compared to A-0. But incorporation of 36 wt% starch in alginate-CNC-lecithin microbeads (ACLS-2) couldn't improve the viability during complete transition. From these results, it was demonstrated that the AC-3 and ACL-1 formulations showed better protection of lactic acid probiotics during sequential transition.

In intestinal fluid, bile salt is the main compound for the destruction of lactic acid bacteria. Upon exposure of the bacteria to bile salts, cellular homeostasis disruptions causes the dissociation of the lipid bilayer and integral protein of their cell membranes, resulting in the leakage of bacterial content and ultimately cell death (Sahadeva *et al.*, 2011; Iyer and Kailasapathy, 2005; Chandramouli *et al.*, 2004). Donovan and Jackson (1993) reported that bile salts mixed with different micelles, which is characterized by an intermixed micellar concentration (IMMC), is

primarily responsible for driving the bile salts into lipid bilayers (e.g. cellular membrane). In our study, lecithin with alginate-CNC (ACL-1) was successfully demonstrated to disrupt bile salts, thus facilitating the survival of probiotics against such challenge. The presence of lecithin allows a spontaneous absorption of bile salt molecules, which increases formation of rod-like hybrid micelles once lecithin reaches saturation with bile salts. The formation of the hybrid micelles with lecithin vesicles is believed to reduce the bile salt IMMC down to a less toxic level. It therefore exerts a protective effect by relieving the bile salt-induced disruption of the plasma membrane (Chen *et al.*, 2012).

5.7.7 Scanning Electron Microscopy (SEM) Analysis

The freeze-drying process altered the structure of the microbeads and there was a difference observed by addition of different additives. **Figure-5.8** shows the morphological characteristics of freeze dried A-0, AC-1, AC-3, ACL-1 and ACLS-2 beads. The surface of the freeze dried alginate beads (A-0) exhibited a dense microstructure with several wrinkles and cracks. Alginate microbeads also exhibited a cohesive and compact surface arrangement with irregularities that included peaks and troughs. There were a small change observed after incorporation of 5 % w/w CNC in alginate microbeads (AC-1). However, addition of 13 % w/w CNC in alginate microbeads (AC-3) reduced the surface wrinkles and cracks with no agglomerations or defects, which was an indication of good miscibility of the CNC with the alginate matrix. Other authors have reported similar surface microstructures of alginate-CNC nanocomposite fibres (Urena-Benavides *et al.*, 2010; Liu *et al.*, 2004). Zohar-Perez *et al.* (2004) also stated that the incorporation of nanomaterial smoothes the surface of alginate beads after freeze-drying. The more smooth and homogeneous surface was observed after addition of 2 % w/w lecithin in alginate-CNC microbeads (ACL-1) which represented a consistent microstructure. Addition of 36 % w/w starch in alginate-CNC-

lecithin showed a congested microstructure that exhibited shrinking on the surface. The surface of all beads contains striations that were attributed to roughness increasing during the drying process and the increased cohesions might be because of the ionic interaction between COO^- (alginate) and Ca^{2+} (CaCl_2). The presence of filler (CNC) and additive (lecithin) had the ability to improve the microbead quality and thus improve the stability of probiotics compared to alginate alone during storage and transition through the gastric passage (Donthidi *et al.*, 2010).

5.8 Conclusions

The world market for functional foods and natural health products has been expanding and is driven by demographic, economic and social trends. Our research successfully developed a novel dry probiotic microcapsule by utilizing CNC. This is the first study to demonstrate the application of CNC in probiotic encapsulation. This developed formulation containing alginate, CNC and lecithin (ACL-1) showed a protective effect for probiotics at both temperatures (25 and 4°C) and also during transition through the gastric passage. Results demonstrated that ACL-1 increased by 61 and 44% the viability of *L. rhamnosus* during storage up to 6 weeks at 25 and 4°C, respectively as compare to alginate matrix alone. It is a promising alternative to generate dried probiotic microbeads for the application of non-refrigerated, long shelf-life food and pharmaceutical products. Our results showed that CNC could be the first and novel cellulosic nanomaterial having the potential to be effective for probiotic encapsulation. Finally, it is interesting to say that the application of CNC in food could link the forest and food industry by developing the world's economy.

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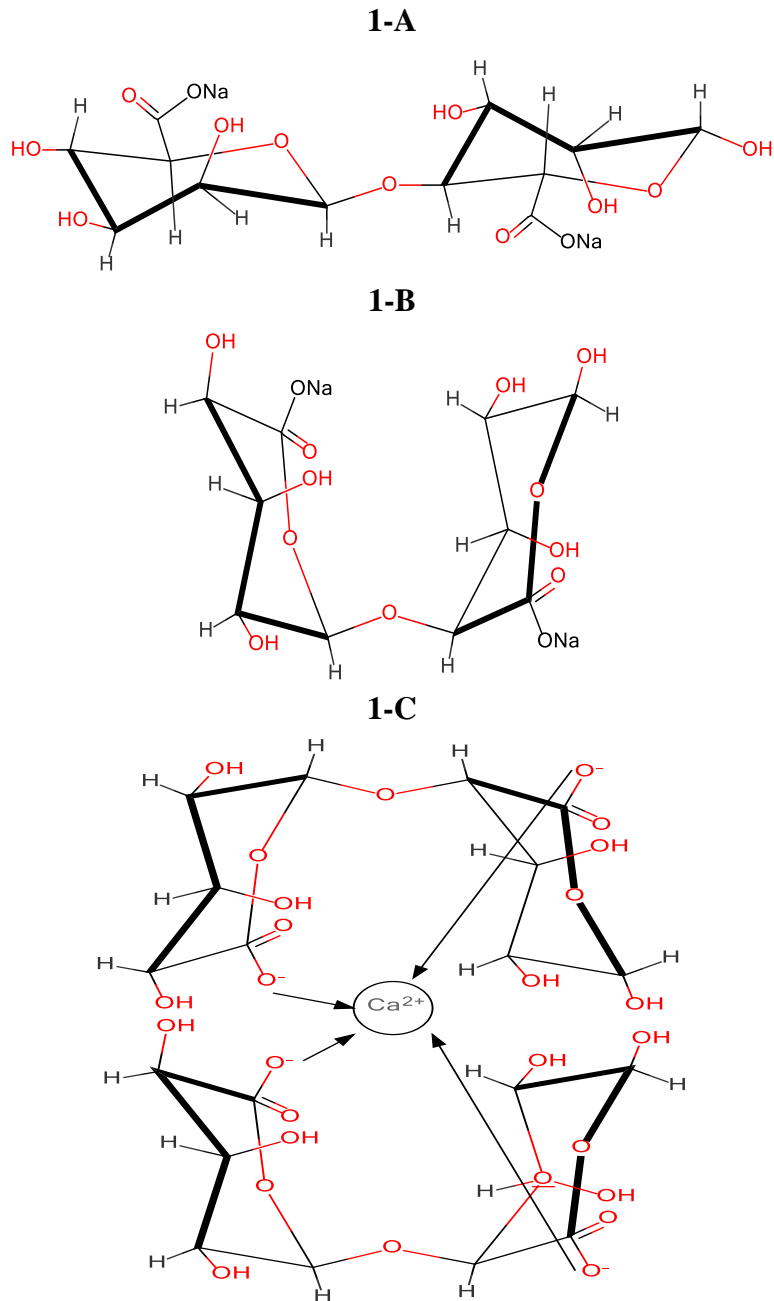


Figure 5.1: A) D-mannuronic acid (M), B) L-guluronic acid (G) (George and Abraham, 2006) and C) egg-box structure of alginate as proposed by Li *et al.*, 2007.

Table-5.1: Optimized formulations are presented according to wt%.

Formulation	Alginate: CNC: lecithin: starch (% w/w)
A-0	100 : 0 : 0 : 0
AC-1	95 : 5 : 0 : 0
AC-3	87 : 13 : 0 : 0
ACL-1	85 : 13 : 2 : 0
ACLS-2	54 : 8 : 2 : 36

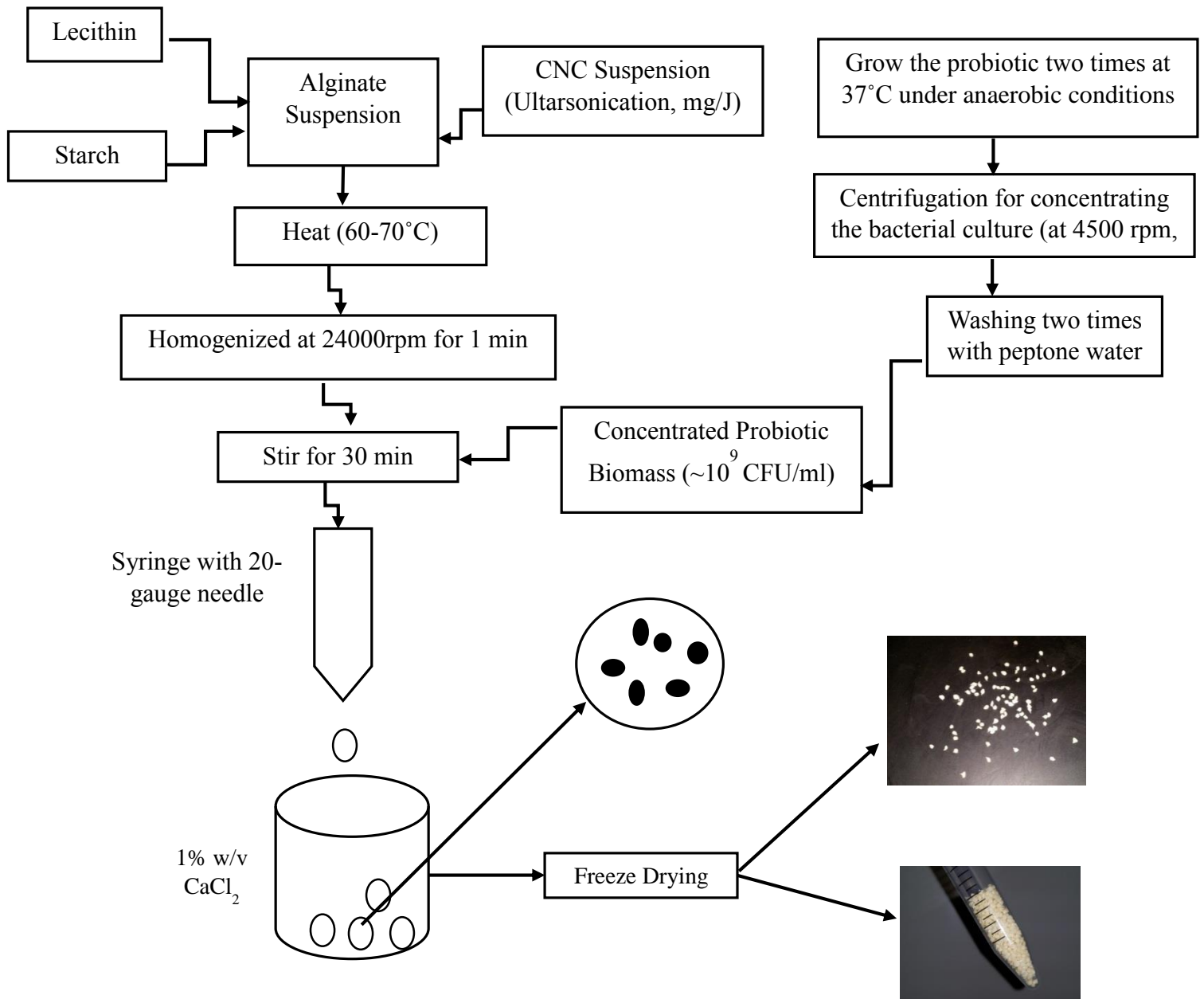


Figure 5.2: General microencapsulation flow diagram for *L.rhamosus* ATCC 9595.

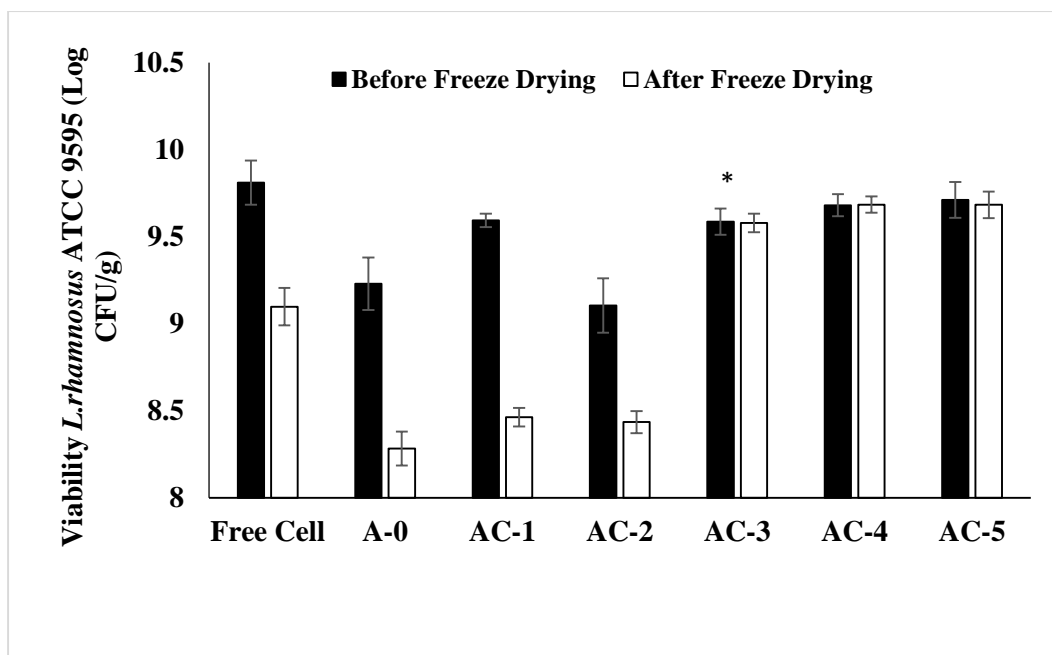


Figure 5.3A: Effect of CNC (% w/w) with alginate beads on the viability of *L. rhamnosus* ATCC 9595 during freeze drying.

A-0: 100 % w/w alginate; AC-1: 5 % w/w CNC + 95 % w/w alginate; AC-2: 10 % w/w CNC + 90 % w/w alginate; AC-3: 13 % w/w CNC + 87 % w/w alginate; AC-4: 17 % w/w CNC + 83 % w/w alginate and AC-5: 20 % w/w CNC + 80 % w/w alginate.

* No significant difference ($P > 0.05$) was found for the samples AC-3, AC-4 and AC-5 between before and after freeze drying treatment as determined by the Student t-test.

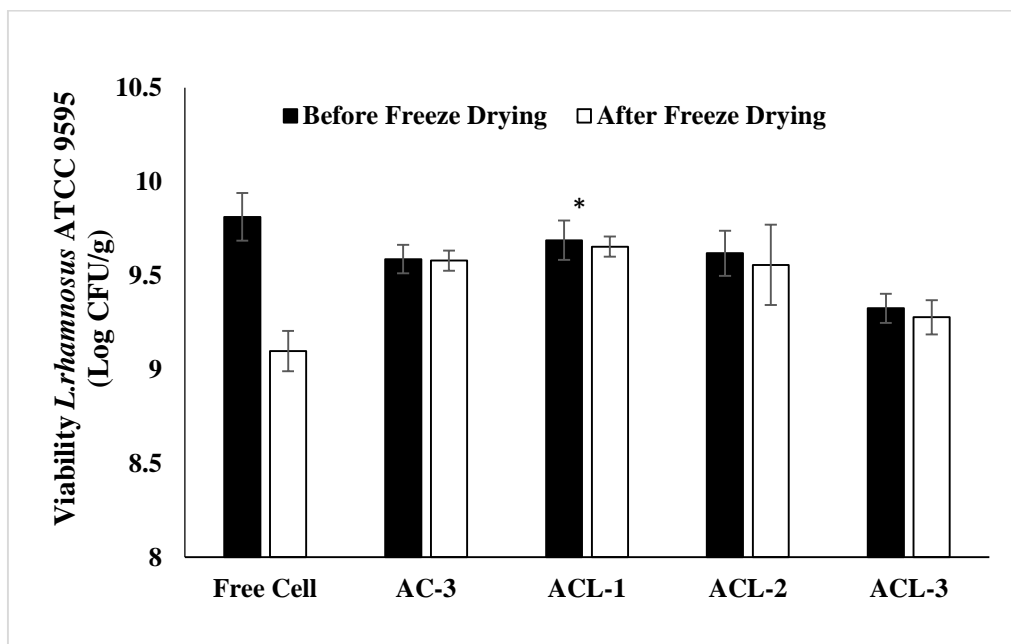


Figure 5.3B: Effect of lecithin (% w/w) on the viability of *L.rhannosus* ATCC 9595 in optimized alginate + CNC beads during freeze drying.

AC-3: 13 % w/w CNC + 87 % w/w alginate; ACL-1: 13 % w/w CNC + 85 % w/w alginate + 2 % w/w lecithin; ACL-2: 11 % w/w CNC + 76 % w/w alginate+ 13 % w/w lecithin; ACL-3: 11 % w/w CNC+ 67 % w/w alginate + 22 % w/w lecithin.

* No significant difference ($P > 0.05$) was found for the samples ACL-1, ACL-2 and ACL-3 between before and after freeze drying treatment as determined by the Student t-test.

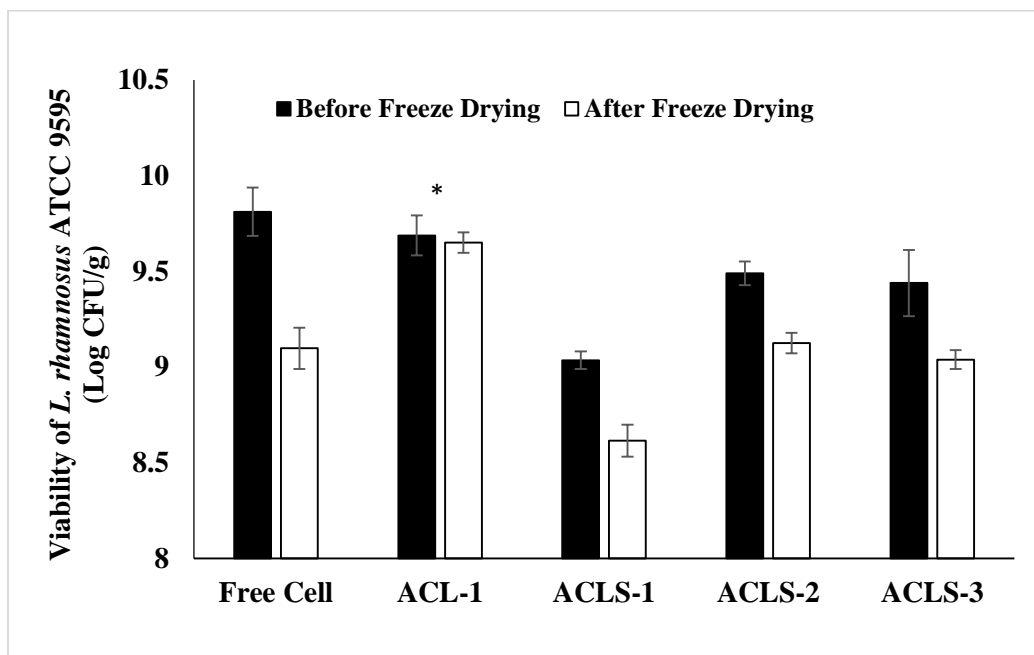


Figure 5.3 (C): Effect of Starch (% w/w) on the viability of *L.rhamnosus* ATCC 9595 in optimized alginate + CNC+ lecithin beads during freeze drying.

ACL-1: 13 % w/w CNC+ 85 % w/w alginate+ 2 % w/w lecithin; ACLS-1: 10 % w/w CNC+ 66 % w/w alginate+ 2 % w/w lecithin+ 22 % w/w starch; ACLS-2: 8 % w/w CNC+ 54 % w/w alginate+ 2 % w/w lecithin+ 36 % w/w starch; ACLS-3: 6 % w/w CNC+ 40 % w/w alginate+ 1 % w/w lecithin+ 53 % w/w starch.

* No significant difference ($P > 0.05$) was found for the samples ACLS-1, ACLS-2 and ACLS-3 between before and after freeze drying treatment as determined by the Student t-test.

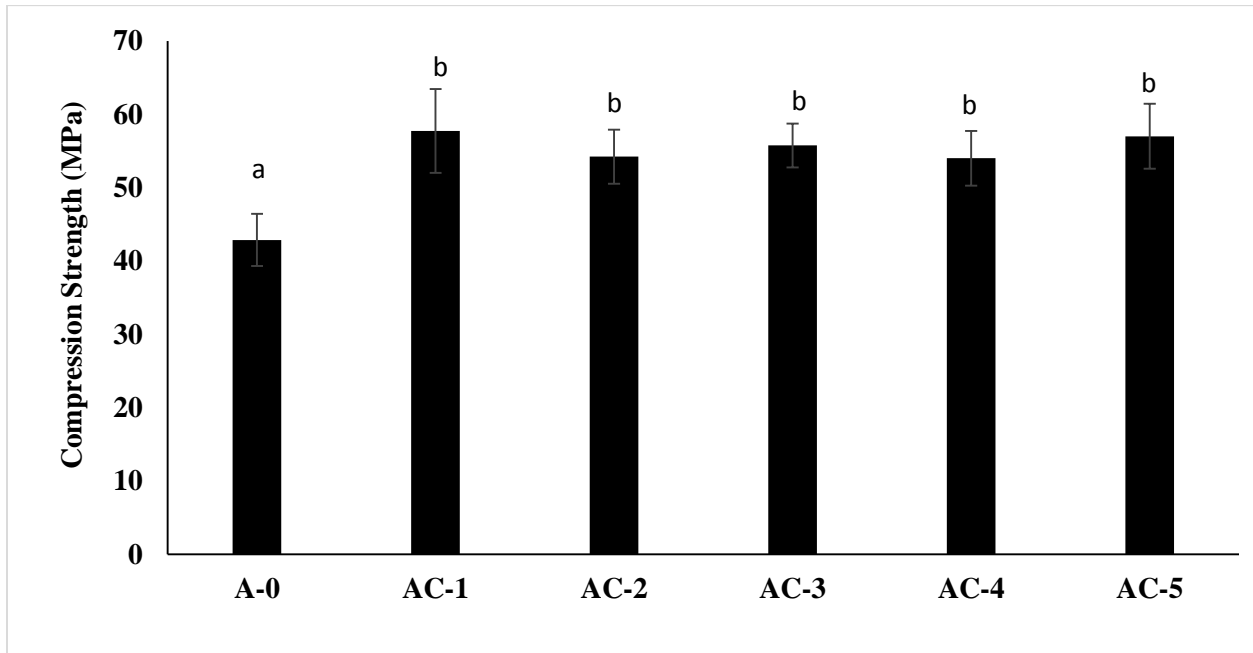


Figure 5.4A: Effect of CNC content (% w/w) on compression strength (MPa) of 100 % w/w (3% w/v) alginate beads, as a function of CNC content in dry matrix. Different lowercase letters indicate the significant differences among the values determined by multiple comparison test (Duncan, $P \leq 0.05$).

A-0: 100 % w/w alginate; AC-1: 5 % w/w CNC + 95 % w/w alginate; AC-2: 9 % w/w CNC + 91 % w/w alginate; AC-3: 13 % w/w CNC + 87 % w/w alginate; AC-4: 17 % w/w CNC + 83 % w/w alginate and AC-5: 20 % w/w CNC + 80 % w/w alginate.

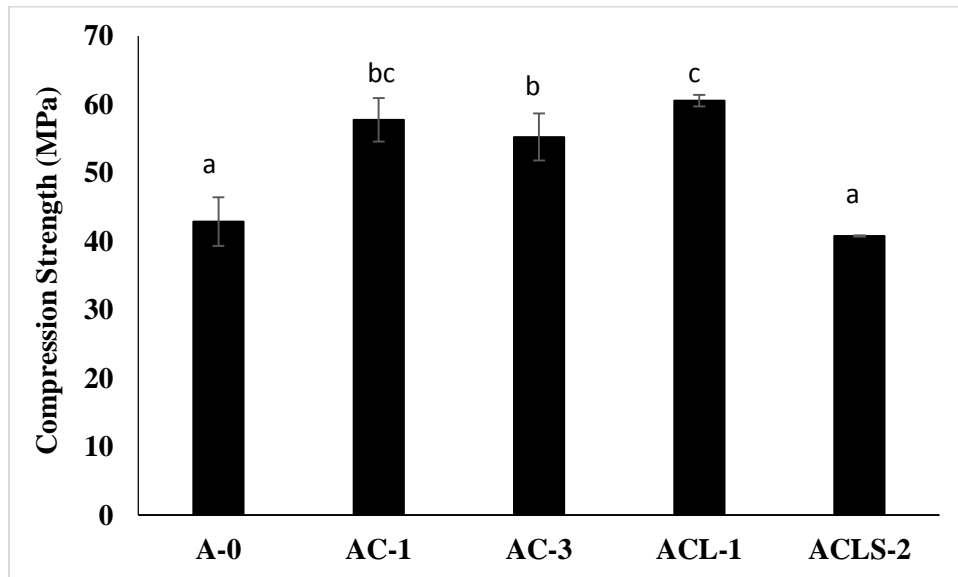
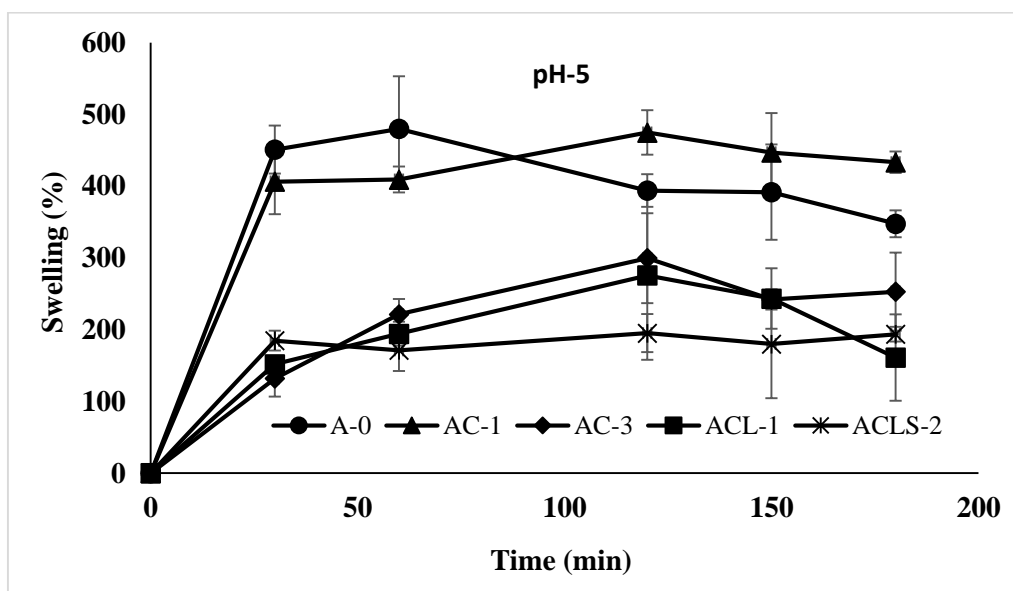
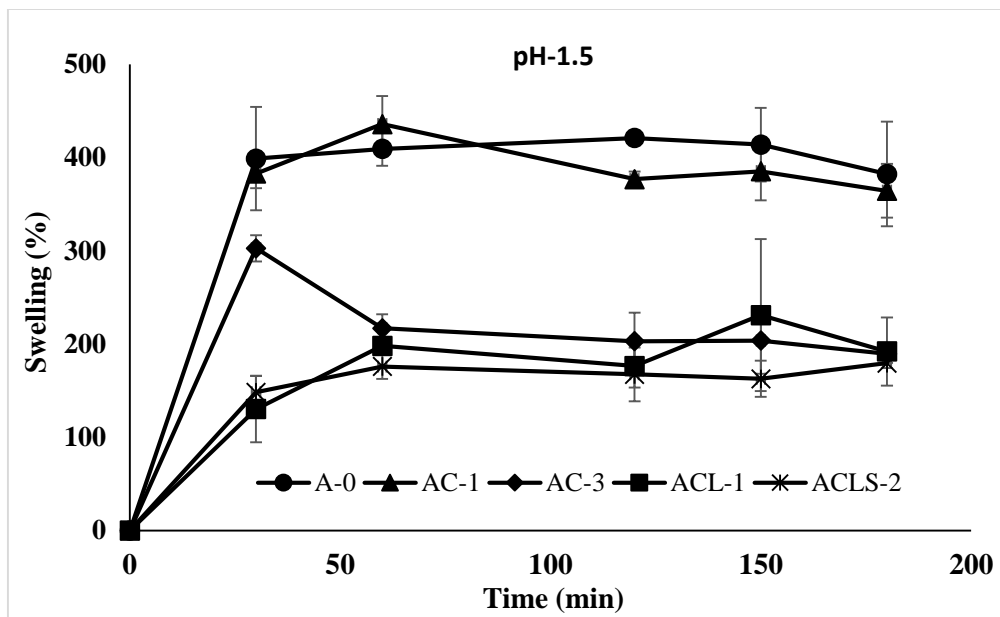


Figure 5.4 (B): Compression strength (MPa) of the optimized formulations. Different lowercase letters indicate the significant differences among the values determined by multiple comparison test (Duncan, $P \leq 0.05$).

A-0: 100 % w/w alginate; AC-1: 95 % w/w alginate + 5 % w/w CNC; AC-3: 87 % w/w alginate + 13 % w/w CNC; ACL-1: 85 % w/w alginate + 13 % w/w CNC + 2 % w/w lecithin; ACLS-2: 54 % w/w alginate + 8 % w/w CNC + 2 % w/w lecithin + 36 % w/w starch.



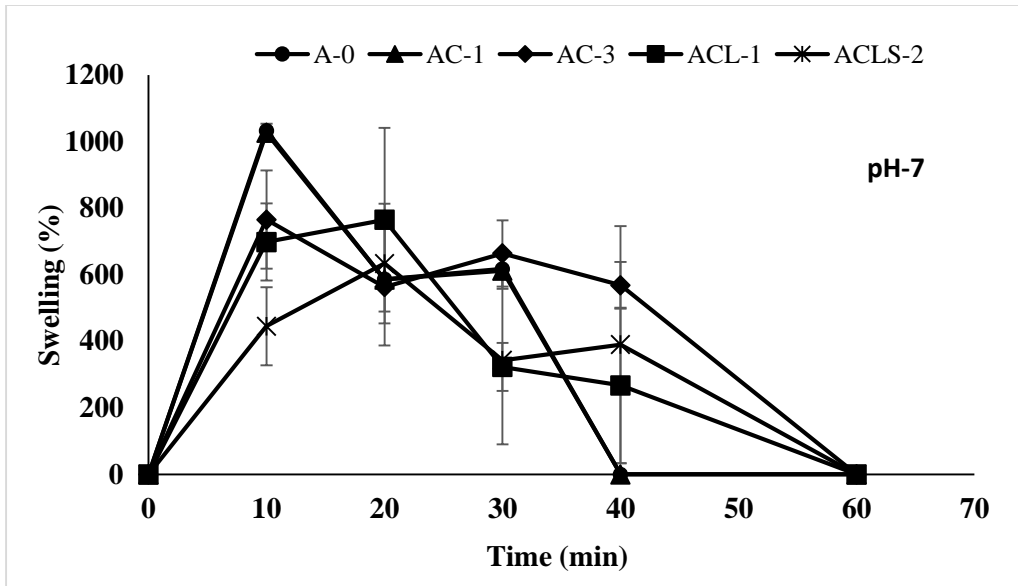


Figure 5.5: Swelling behavior of freeze-dried optimized beads at two different simulated gastric fluid pH (1.5 and 5) values and in simulated intestinal fluid (pH 7).

A-0: 100 % w/w alginate; AC-1: 95 % w/w Alginate+5 % w/w CNC; AC-3: 87 % w/w Alginate+13 % w/w CNC; ACL-1: 85 % w/w alginate + 13 % w/w CNC + 2 % w/w lecithin; ACLS-2: 54 % w/w alginate + 8 % w/w CNC + 2 % w/w lecithin + 36 % w/w starch.

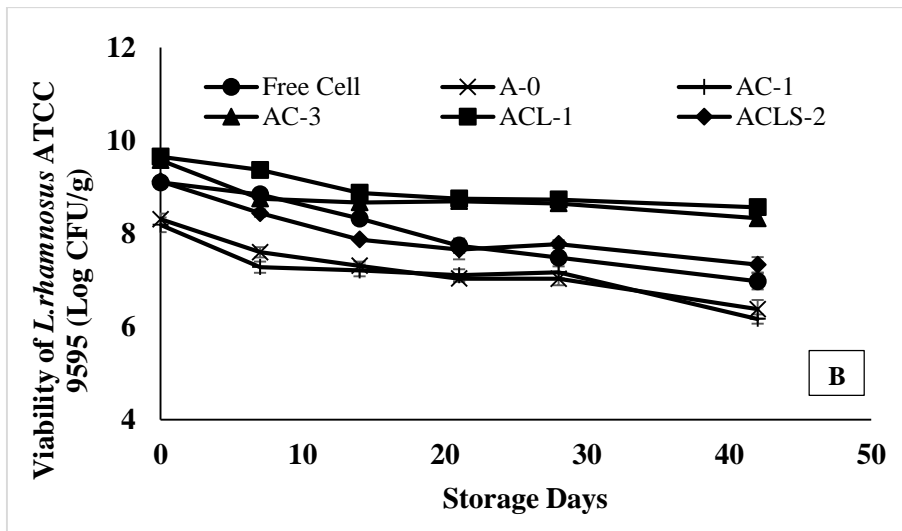
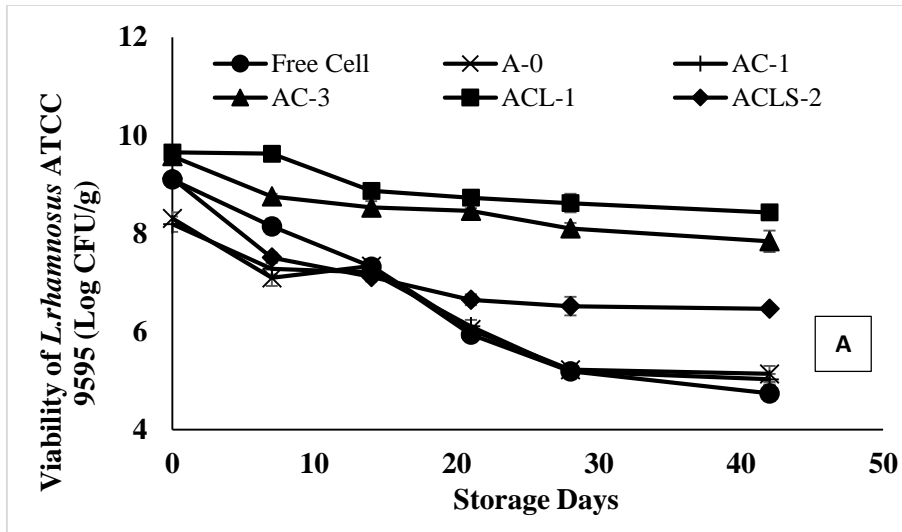


Figure 5.6: The storage stability of freeze-dried free and microencapsulated *L. rhamnosus* **A)** at 25° C and **B)** 4° C.

A-0: 100 % w/w alginate; AC-1: 95 % w/w Alginate+5 % w/w CNC; AC-3: 87 % w/w Alginate+13 % w/w CNC; ACL-1: 85 % w/w alginate + 13 % w/w CNC + 2 % w/w lecithin; ACLS-2: 54 % w/w alginate + 8 % w/w CNC + 2 % w/w lecithin + 36 % w/w starch.

Table 5.2: Inactivation rate constants k (d^{-1}) for *L. rhamnosus* at 25 and 4 °C storage conditions. The determination coefficients (R^2) of each individual k value are given in brackets next to the respective k -value.

	25° C	4° C
	k (R^2)	k (R^2)
Free Cell	0.11 (0.93)	0.05 (0.97)
A-0	0.08 (0.87)	0.03 (0.92)
AC-1	0.08 (0.91)	0.03 (0.82)
AC-3	0.04 (0.88)	0.01 (0.70)
ACL-1	0.03 (0.83)	0.02 (0.83)
ACLS-2	0.06 (0.70)	0.03 (0.82)

Table 5.3 A: Water activity of freeze-dried *L.rhamnosus* free cell and microencapsulated *L.rhamnosus* samples during storage at 25°C*.

Formulation	Day-0	Day-14	Day-28	Day-42
Free Cell	0.05±0.01 ^{aA}	0.15±0.04 ^{bBC}	0.25±0.04 ^{cBC}	0.29±0.03 ^{dB}
A-0	0.14±0.04 ^{aC}	0.25±0.04 ^{bE}	0.31±0.06 ^{bcC}	0.33±0.06 ^{dB}
AC-1	0.12±0.02 ^{aBC}	0.17±0.06 ^{abCD}	0.23±0.04 ^{bB}	0.29±0.08 ^{cB}
AC-3	0.09±0.03 ^{aB}	0.08±0.02 ^{aA}	0.07±0.04 ^{aA}	0.11±0.05 ^{aA}
ACL-1	0.07±0.01 ^{aA}	0.11±0.05 ^{abAB}	0.09±0.05 ^{abA}	0.12±0.05 ^{bA}
ACLS-2	0.13±0.04 ^{aBC}	0.22±0.05 ^{bDE}	0.26±0.06 ^{bBC}	0.26±0.06 ^{bB}

Table 5.3B: Water activity of freeze-dried *L.rhamnosus* free cell and microencapsulated *L.rhamnosus* samples during storage at 4°C*.

Formulation	Day-0	Day-14	Day-28	Day-42
Free Cell	0.05±0.01 ^{aA}	0.09±0.02 ^{bA}	0.15±0.04 ^{cAB}	0.18±0.04 ^{cB}
A-0	0.16±0.04 ^{aC}	0.16±0.05 ^{bB}	0.20±0.06 ^{abC}	0.24±0.04 ^{bC}
AC-1	0.11±0.04 ^{aB}	0.10±0.02 ^{aA}	0.18±0.03 ^{bBC}	0.21±0.03 ^{cBC}
AC-3	0.11±0.03 ^{aB}	0.09±0.02 ^{aA}	0.10±0.03 ^{aA}	0.10±0.04 ^{aA}
ACL-1	0.06±0.01 ^{aA}	0.08±0.02 ^{aA}	0.10±0.06 ^{aA}	0.09±0.06 ^{aA}
ACLS-2	0.12±0.04 ^{aB}	0.18±0.07 ^{abB}	0.21±0.04 ^{bC}	0.23±0.04 ^{bC}

*Values are means ± standard deviations. Within each row, means with the same lowercase letters are not significantly different (P>0.05) and within each column, means with the same uppercase letters are not significantly different (P>0.05) as determined by multiple comparison test (Duncan).

Table 5.4: Survival of *L. rhamnosus* ATCC 9595 (Log CFU/g) after 2 h SGF treatment.* & **

Formulation	Beads before freeze-drying		Beads after freeze-drying	
	Before SGF (2 h) treatment	After SGF (2 h) treatment	Before SGF (2 h) treatment	After SGF (2 h) treatment
Free Cell	9.81±0.13 ^c	2.00±0.00 ^a	9.03±0.13 ^c	2.00±0.00 ^a
A-0	9.21±0.15 ^a	7.76±0.43 ^b	8.27±0.10 ^a	6.78±0.04 ^b
AC-1	9.59±0.04 ^b	8.03±0.40 ^b	8.46±0.05 ^b	6.89±0.05 ^c
AC-3	9.58±0.08 ^b	8.73±0.16 ^c	9.58±0.05 ^e	8.65±0.07 ^e
ACL-1	9.68±0.10 ^b	8.86±0.03 ^c	9.65±0.05 ^e	8.71±0.09 ^e
ACLS-2	9.61±0.08 ^b	8.57±0.05 ^c	9.12±0.05 ^d	7.56±0.09 ^d

*Means within a column bearing the same letter were not significantly different ($P>0.05$) as determined by multiple comparison test (Duncan).

**Significant difference ($P\leq 0.05$) was found between before and after SGF (2 h) treatment samples as determined by the Student t-test.

A-0: 100 % w/w alginate; AC-1: 95 % w/w Alginate+5 % w/w CNC; AC-3: 87 % w/w Alginate+13 % w/w CNC; ACL-1: 85 % w/w alginate + 13 % w/w CNC + 2 % w/w lecithin; ACLS-2: 54 % w/w alginate + 8 % w/w CNC + 2 % w/w lecithin + 36 % w/w starch.

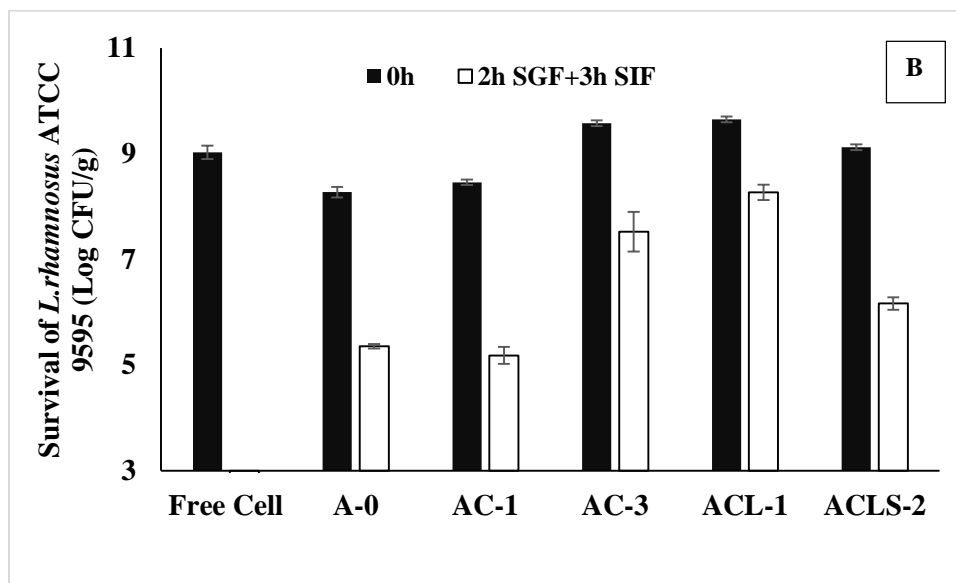
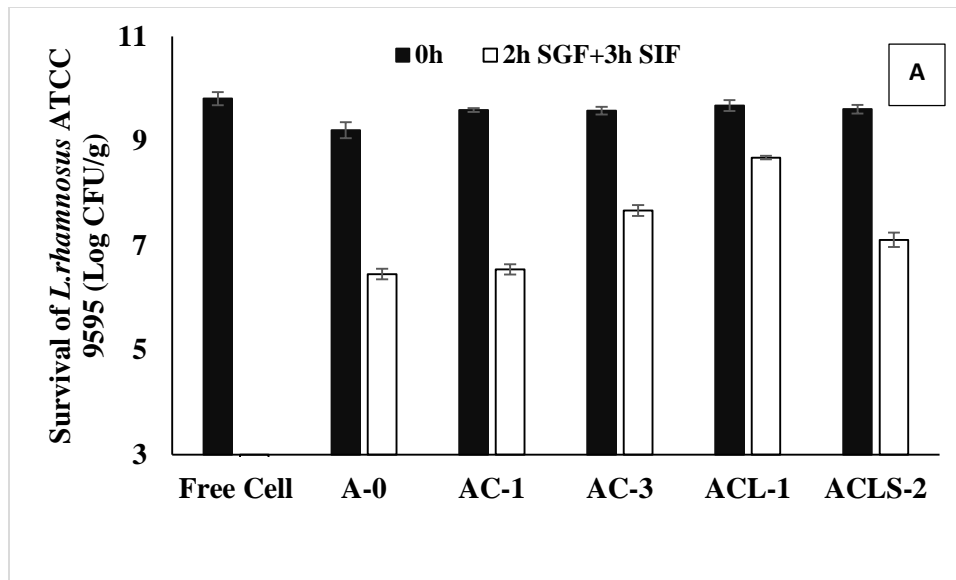
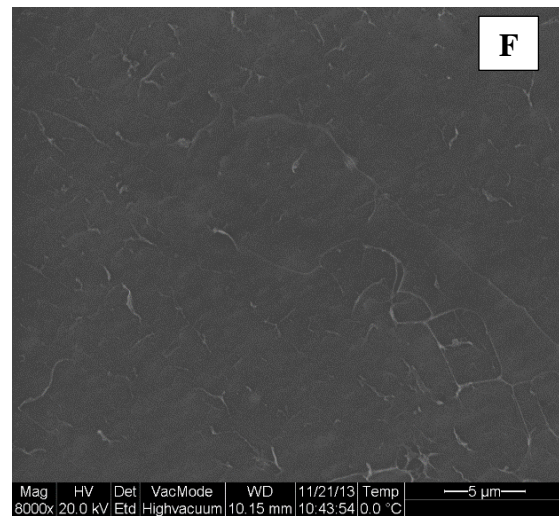
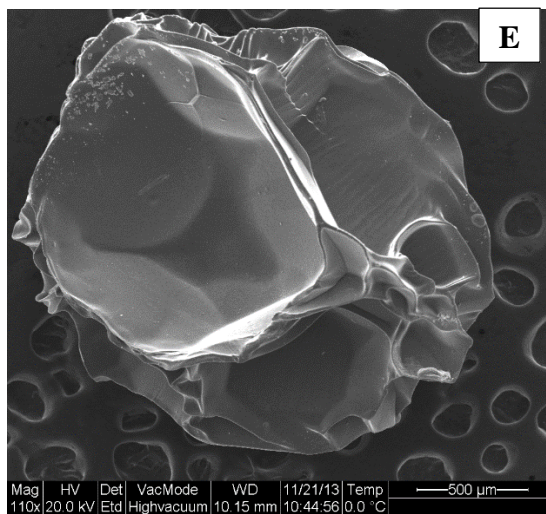
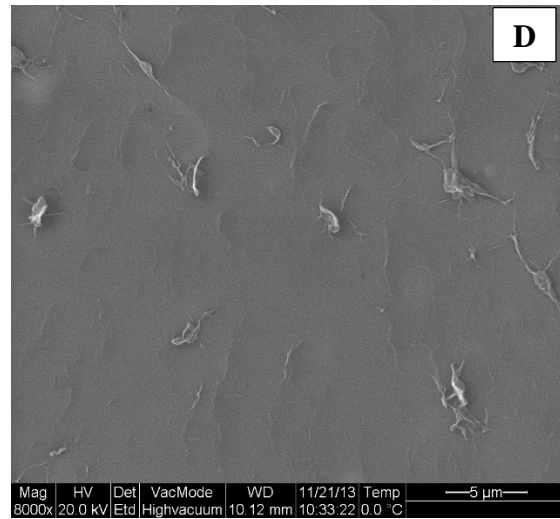
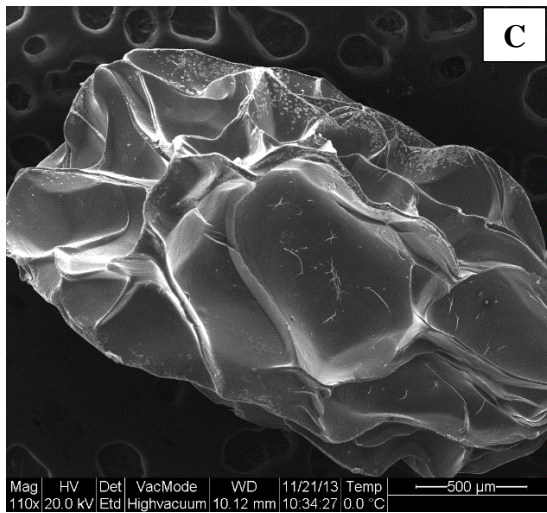
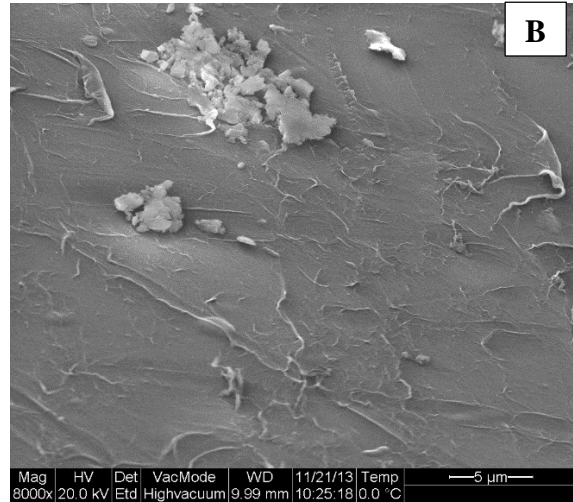
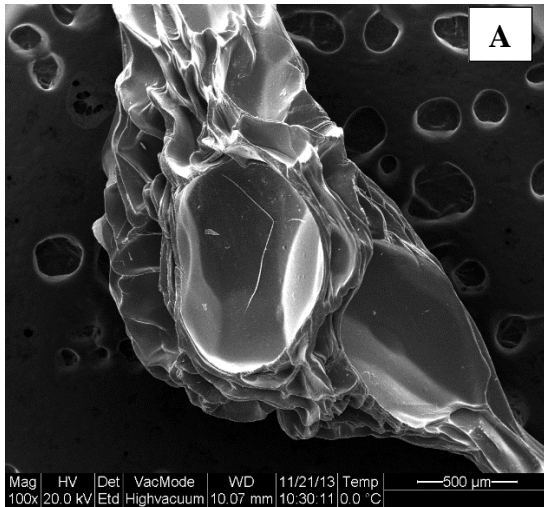


Figure 5.7: Survival of *L.rhamnosus* ATCC 9595 in optimized microbead formulations after exposure in SGF (2 h) + SIF (3 h) **A)** before freeze-drying and **B)** after freeze-drying.

A-0: 100 % w/w alginate; AC-1: 95 % w/w Alginate+5 % w/w CNC; AC-3: 87 % w/w Alginate+13 % w/w CNC; ACL-1: 85 % w/w alginate + 13 % w/w CNC + 2 % w/w lecithin; ACLS-2: 54 % w/w alginate + 8 % w/w CNC + 2 % w/w lecithin + 36 % w/w starch.



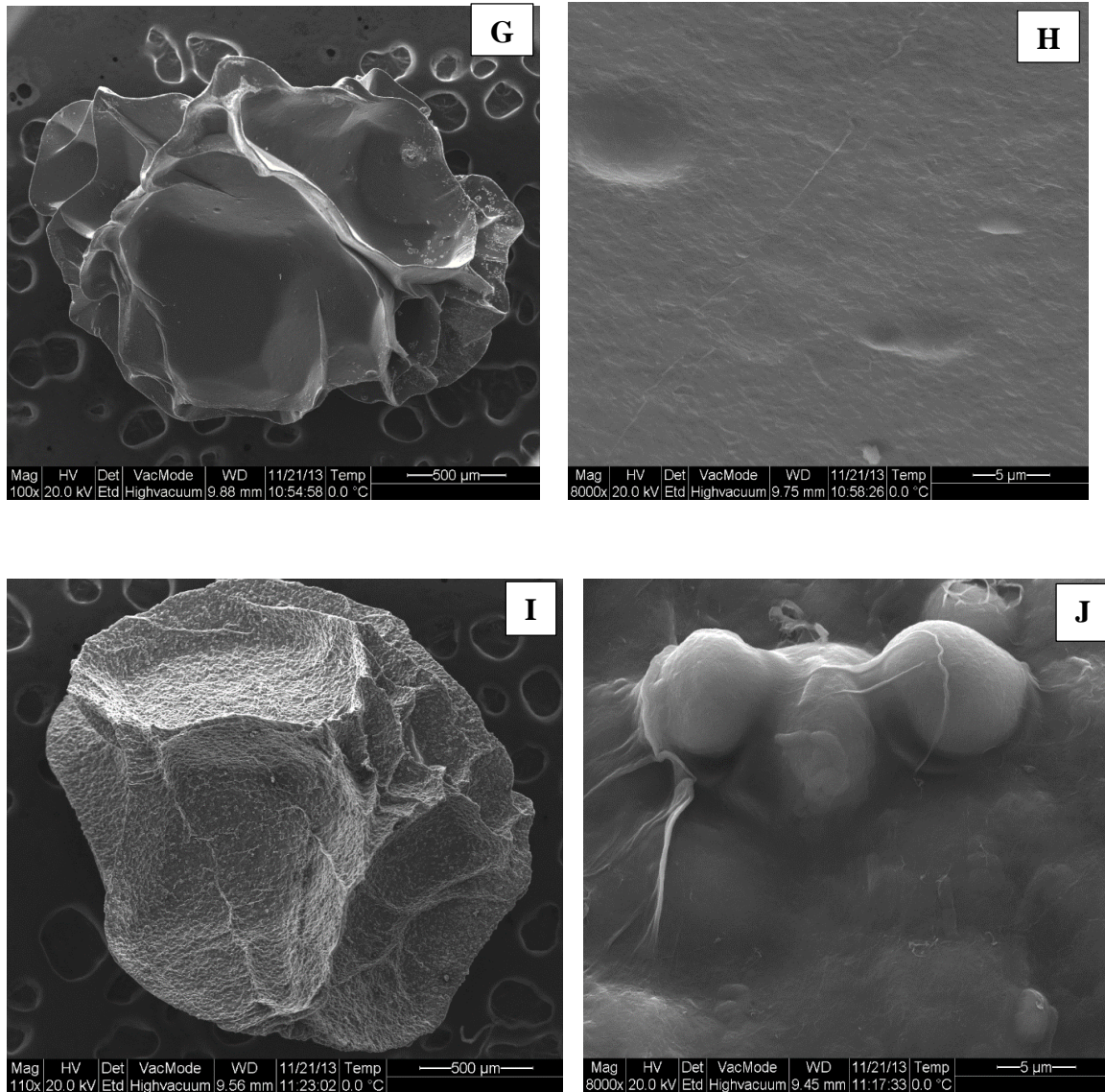


Figure 5.8: Surface SEM micrographs of freeze-dried beads of (A) and (B) A-0: 100 % w/w alginate; (C) and (D) AC-1: 95 % w/w alginate + 5 % w/w CNC; (E) and (F) AC-3: 87 % w/w alginate + 13 % w/w CNC; (G) and (H) ACL-1: 85 % w/w alginate +13 % w/w CNC + 2 % w/w lecithin; (I) and (J) ACLS-2: 54 % w/w alginate + 8 % w/w CNC + 2 % w/w lecithin + 36 % w/w starch, respectively.

5.11 General Discussions of the Pathent Application

Microencapsulation matrix developed in this research enhanced the viability of probiotic during storage and under treatment in gastro-intestinal tract. Microencapsulation matrix ACL-1 improved the viability of probiotic by 44 and 61% during storage at 4 and 25° C, respectively, compared to control (A-0). ACL-1 also increased the viability of probiotic by 52% under passage through the gastrointestinal tract. This study revealed that addition of 13 % w/w CNC and 2 % w/w lecithin in alginate microbead showed an improvement on viability of probiotic.

Chapter-6

Patent Application

Development of Probiotic Tablet Formulation with Cellulose NanoCrystals (CNC) by using Central Composite Design (CCD)

Tanzina Huq¹, Khanh Dang Vu¹, Bernard Riedl², Jean Bouchard³ and Monique Lacroix^{1*}

¹Research Laboratories in Sciences Applied to Food, Canadian Irradiation Centre (CIC),
INRS-Institut Armand-Frappier, Université du Québec, 531 Boulevard des Prairies,

Laval, Québec, H7V 1B7, Canada

²Département des sciences du bois et de la forêt, Faculté de foresterie, géographie et géomatique,

Université Laval, Québec, G1V 0A6, Canada

³FPInnovations, 570 Boulevard St. Jean, Pointe-Claire, Québec, H9R 3J9, Canada

* **Corresponding Author:** Prof. Monique Lacroix

Telephone: +1-450-687-5010; Fax: +1-450-686-5501; **E-mail:** monique.lacroix@iaf.inrs.ca

6.1 Contribution to the Authors

This article is in preparation for patent application. The whole experimental work, data analysis and draft have been prepared by Tanzina Huq under the direction of Prof. Monique Lacroix. Prof. Lacroix has corrected the protocol and also helped me to solve the problems during experiments. Prof. Lacroix and Prof. Bernard Riedl (co-director) have corrected the draft and improved the discussion of publications. Dr. Khanh Dang Vu helped me to design the central composite design. Dr. Jean Bouchard has corrected the draft.

6.2 Specific Objectives of the Patent Application

This study demonstrated the microencapsulating matrix formulation development for probiotic in dry tablet form. The formulation developed in Publication-4 was not able to keep the viability of probiotic at 25°C for more than 2 months. The main objectives of this were to improve the viability of probiotic under gastric treatment and during storage at 25°C. This formulation was developed by response surface methodology (RSM). RSM permits an analysis of multiple factors and eliminates the need for a large number of experimental runs that are otherwise required in a conventional one-factor-at-a-time approach.

6.3 Résumé

Un modèle expérimental statistique de type "Conception composite centrale ou CCD" a été utilisé pour aider à développer et optimiser une formule probiotique par l'évaluation de la viabilité (%) lors de l'exposition sous milieu gastrique (2h). Il a été constaté que, selon le CCD, la formulation de comprimés probiotiques optimisé contenant un alginat, de la pectine et des CNC a montré 84% de viabilité après un passage séquentiel à travers le tractus gastro-intestinal tandis que les comprimés probiotiques préparés individuellement avec de l'alginat, de la pectine et des CNC n'a montré que 19, 17 et 10%, respectivement, de viabilité des probiotiques. Une étude du gonflement a également révélé que le CCD a permis d'optimiser une tablette probiotique, laquelle a subi moins de dissolution à un pH gastrique de 1.5 et une dissolution continue à pH intestinal de 7. La tablette probiotique optimisée à l'aide de la CCD a été stockée à 25 et 4 ° C jusqu'à 42 jours. Il a été constaté qu'après 42 jours de stockage, cette formulation appliquée aux comprimés a donné des viabilités de 8.55 et 8.81 log UFC /g à 25 et 4° C, respectivement.

6.4 Abstract

Central composite design (CCD) was used to develop a probiotic formulation by evaluating the viability (%) during exposure under gastric medium (2h). It was found that CCD optimized probiotic tablet formulation containing alginate, pectin and CNC showed 84% viability after sequential transition through the gastrointestinal tract whereas the probiotic tablet individually prepared with alginate, pectin and CNC showed only 19, 17 and 10 % viability of probiotic, respectively. Swelling study also revealed that CCD optimized probiotic tablet underwent less dissolution at gastric pH -1.5 and a continuous dissolution at intestinal pH -7. CCD optimized probiotic tablet was stored at 25 and 4° C up to 42 days. It was found that after 42 days of storage, this formulation had 8.55 and 8.81 log CFU/tablet viability at 25 and 4° C, respectively.

Keywords: Cellulose Nanocrystal, Pectin, Alginate, Probiotic Tablet, Central composite design.

6.5 Introduction

Global interest is linked to the numerous studies that have attributed, and continued to attribute several health benefits related to probiotic-containing products. Probiotics are defined as a live microbial food supplement which beneficially affects the host by improving its intestinal microbial balance. These beneficial actions range between the alleviation of lactose intolerance, the reduction of symptoms caused by viral and antibiotic associated diarrhea, the modulation of the immune system, the prevention of inflammatory bowel disease and the reduction of risks associated with mutagenicity and carcinogenicity (Saxelin *et al.*, 2005; Vasiljevic and Shah, 2008). Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) define probiotics as “Live microorganisms (bacteria or yeasts), which when ingested or locally applied in sufficient numbers confer one or more specified demonstrated health benefits for the host”(FAO/WHO, 90 2001). Lactic acid bacteria (LAB) are the most important probiotic microorganisms typically associated with the human gastrointestinal tract (Huq *et al.*, 2013).

Now-a-days, functional food products containing specific probiotic strains are developed in different formulations, such as fermented milk (Lavermicocca, 2006), chewing gum (Caglar *et al.*, 2007) and capsules (Bruno and Shah, 2003). But these products showed limited shelf life stability due to their high water activity (a_w). Probiotics are very sensitive at high water activity and low pH level. Porubcan *et al.* (2006) has already demonstrated that microencapsulated *Lactobacillus rhamnosus* strain HN001 with 0.12 a_w dramatically reduced its product shelf life at 25°C as compared with a_w of 0.045. They also reported that at pH 1.6 the viability of *L. rhamnosus* strain HN001 decreased below 10^4 CFU/ml after 90 min exposure under simulated gastric fluid (SGF). Due to this reason, there is a prerequisite for the development of dry formulations that can protect

the probiotic bacteria from the harsh conditions in the stomach and simultaneously during storage at room temperature. In the pharmaceutical field, acid labile drugs are formulated in tablets which are able to protect them from these harsh conditions and also can have very low a_w . The tablet can deliver the active substances into the intestinal tract without losing its activity and the product can retain a longer shelf life during storage at room temperature. Tablets can be easily designed to control the release and enhance the adhesion and colonization of the probiotic microorganisms to the epithelial mucosa of the human host by using the proper kinds of tablet excipients (Maggi *et al.*, 2000). The probiotic tablets with suitable excipients and optimum compression force were reported to ensure high stability of *L. acidophilus* under artificial gastric juice (Stadler and Viernstein, 2003). In addition, tablets have advantages above other dosage forms. These include accurate dosage, ease of administration, good patient acceptance and suitability for large-scale production.

Biopolymers have been used in pharmaceutical industry since last 15 years for tablet development as an excipient. Alginate is one of the most popular biopolymers used for probiotic encapsulation (Huq *et al.*, 2013). It is a linear polysaccharide consisting of β -(1 \rightarrow 4)-linked D-mannuronic acid (M) and α -(1 \rightarrow 4)-linked L-guluronic acid (G) residues derived from brown algae or bacterial sources. The naturally occurring alginate polymer has long been used in the food and beverage industries as thickening, gel-forming and colloidal stabilizing agents (Khan *et al.*, 2010). They are also used as binders and disintegrants in tablet manufacture. Alginate rapidly forms a viscous solution or gel in contact with aqueous medium which can act as a diffusion barrier for the active compound such as probiotics (Liew *et al.*, 2006; Sriamornsak *et al.*, 2007). Pectin could be another important biopolymer for probiotic tablet development. It is a linear biopolymer of D-galacturonic acid units and their methyl esters connected with α -(1, 4)-glycosidic bonds. The linear structure of

pectin is partly branched with (1, 2)-linked side chains consisting of L-rhamnose residues and some other neutral sugars. It is an important water-soluble polysaccharide of plant origin and is of considerable interest for the food industry as a gelling agent and a stabilizer in jams, fruit jellies, yogurt drinks and lactic acid beverages. In addition to being a widely used food additive, alginate and pectin possess several characteristics that make them a potential biopolymer suitable for the development of control-release systems for colon delivery (Ahrabi *et al.*, 2000; Sriamornsak *et al.*, 2007). Kim *et al.* (1998) reported that alginate and pectin exhibited a good resistance to consolidation because of a high elastic recovery during compression and ejection. The mechanism of the compaction is suggested to be fragmentation with little plastic deformation. Ahrabi *et al.* (2000) has suggested microcrystalline cellulose (MCC) as a plastically deforming directly compressible material to improve the quality of tablets. A current study revealed that utilization of CNC with alginate and pectin was able to improve the plastically deforming and compressibility properties of tablet. CNC is a cellulose based nanomaterial and is composed of rod-like shaped nanoparticles and it can be referred to nanocrystals, whiskers or nanowhiskers. CNC used in this study was obtained by the controlled sulfuric acid hydrolysis of commercial bleached softwood pulp (Dong *et al.*, 1998). It was found that CNC which was formed after sulfuric acid hydrolysis showed a stable chiral nematic crystalline structure in aqueous phase (Revol *et al.*, 1992). These cellulose nanocrystals are featured by an average diameter of 5–10 nm with average length of around 100 nm and are found to be non-toxic (Habibi *et al.*, 2010; Kovacs *et al.*, 2010; Alexandrescu *et al.*, 2013). Kolakovic *et al.* (2011) has used cellulose nanofibre as a tablet excipient for fast disintegrating drug delivery. But for the probiotic tablet market, CNC would be a new and challenging nanomaterial combined with other bio polymeric excipients such as alginate and pectin. The biopolymer (alginate, pectin and CNC) formulations were developed using a three-

factor with five level face-centered central composite design (CCD) to protect the viability of the probiotic at gastric transition. This design is suitable for exploring quadratic response surfaces that permits the development of a polynomial model. CCD has its abilities to estimate second order and third-order effects, to detect inter-relationships between factors and to locate response optima (Late and Bangal, 2010).

Based on this information, we investigated our present study to design the tablet formulations with three different biopolymers (alginate, pectin and CNC) for the protection of lactic acid bacteria during 2h transition through the gastric medium by using CCD and response surface methodology (RSM). The swelling study was carried out to check the CCD optimized tablet matrix property in dissolution medium. The viability of lactic acid bacteria during sequential transition through the gastrointestinal tract (from SGF to SIF) and storage (at 25 and 4°C) was investigated by the CCD optimized formulation.

6.6 Materials and Methods

6.6.1 Materials

Sodium alginate (glucuronic acid content ~ 65 - 70%; mannuronic acid content ~5 - 35%) and Pectin (high methyl ester content) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). CNC was produced by FPInnovations (Pointe-Claire, QC, Canada).

6.6.2 Freeze Dried Probiotic Culture Preparation (Biomass)

L. rhamnosus was obtained from ATCC 9595 (ATCC, Manassas, VA, USA) and stored at -80°C in Lactobacilli MRS broth (Difco Laboratories, Detroit, MI, U.S.A.) supplied with 10% (v/v) glycerol added. *L. rhamnosus* ATCC 9595 was transferred twice in MRS broth at 37°C. Culture cells were harvested after 24 h by centrifugation (4500g, 10 min at 4°C), washed and resuspended in 0.1% (w/v) peptone water to a final concentration of $\sim 10^9$ - 10^{10} colony-forming units CFU/ml.

The harvested probiotic biomass was frozen at $-80\text{ }^{\circ}\text{C}$ for 2 to 3 h. Then the sample was dried by freeze-dryer (Labconco Freeze Dry Systems, U.S.A.) at $-49\text{ }^{\circ}\text{C}$ and 0.09 torr pressure for 48 h (2 days) to obtain water activity, $a_w \sim 0.031$. After freeze drying, the viable concentration of *L. rhamnosus* was again evaluated and was found to be around $\sim 10^9$ CFU/ml.

6.6.3 Probiotic Tablet Preparation Method

Probiotic tablets (different weight, mg according to CCD) were made based on alginate (a_w -0.08), pectin (a_w -0.07) and CNC (a_w -0.05). Probiotic tablets were obtained by direct compression of a homogenous mixture of dry powders containing different polymer excipients (alginate, pectin and CNC) and 30 mg of probiotic *L. rhamnosus* bacteria powder (containing $\sim 10^9$ colony-forming units, CFU/mg). The mixture was compressed at 1.2 T/cm^2 using a manual hydraulic Carver press (Wabash, IN, USA) and 10.0 mm cylinder outfits with flat-faced punches (Calinescu and Mateescu *et al.*, 2008).

6.6.4 Experimental Design

The main concept of this study included 1) experimental design using CCD, 2) preparation of probiotic (*L. rhamnosus*) tablets by mixing three different polymers (alginate, pectin and CNC) according to the CCD and 3) determination of the viability (%) of probiotic in simulated gastric fluid (SGF, 2hr) using CCD and RSM.

6.6.5 Experimental Design for Probiotic Tablet Using CCD

The central composite design contained an imbedded factorial or fractional factorial design with center point that is augmented with a group of star points that allows estimation of curvature. In face-centered central composite design, star points were at the center of each face of the factorial space. The second-order regression models were developed based on the regression analysis of the statistically significant variables (Adjallé *et al.*, 2011). This experiment was designed using a 3

factor CCD with 4 replicates at the center point to build the response surface model. **Table 6.1** represents the principal values of the three independent variables at five levels (-2, -1, 0, 1, 2). The 3 independent variables were the concentration of alginate (X_1), pectin (X_2) and CNC (X_3). Preliminary experiments were carried out to determine the optimal values of the independent variables.

The dependent variable was the viability (%) of *L. rhamnosus* during SGF (2hr) treatment. By measuring the viability (%) of the probiotic, it was possible to establish an optimum formulation with alginate, pectin and CNC concentration for probiotic tablet. The results of the CCD experiments and measurement of the dependent variables (viability, %) were analyzed by STATISTICA 8 of STATSOFT Inc. (Thulsa, US) by RSM. The effects of independent variables on the Y response of the dependent variables were analyzed according to a polynomial model of second order of surface response given by the following general equation:

$$Y = A_0 + \sum_{i=1}^n A_i X_i + \sum_{j \leq i}^n B_{ij} X_i X_j$$

Where Y, predicted response; A_0 , intercept; X_i and X_j , values of various levels of the independent variables; A_i , values of linear coefficients; and B_{ij} , values of quadratic coefficients (Khan *et al.*, 2014).

6.6.6 Viability of *L. rhamnosus* in Probiotic Tablet after Incubation in Simulated Gastric Fluid (SGF)

The SGF was formulated according to United States Pharmacopeia, 1995 and composed of 3.2 g/l of pepsin (approx. 600 units/mg, with one enzyme unit producing a ΔA_{280} of 0.001/min at pH 2.0 and 37 °C, measured as trichloroacetic acid-soluble products from hemoglobin as a substrate) and 2.0 g/l NaCl and pH was finally adjusted to 1.5 using HCl (1.0 M) solution. SGF was sterilized by

filtration with a Bottle Top Vacuum Filter (0.2 μm pore size; Nalge Nunc International, NY, USA). Tablets containing probiotic were placed in a tube containing 10ml of sterile SGF (pH 1.5) and incubated at 37° C for 2h under mild agitation (100 rev/min) in a G24 Environmental Incubator Shaker (New Brunswick Scientific Co., USA). After incubation, the probiotic tablet was removed and dissolved in phosphate buffer (pH 7, 0.5 M). A 1.0-ml aliquot of dissolved tablets was collected and analyzed. The survival of probiotic cells reported as percentage viability was calculated according to the following equation (Klayraung *et al.*, 2009):

$$\text{Viability (\%)} = \frac{\frac{\text{CFU}}{\text{mg}} \text{ Tablet after exposure to the test medium}}{\frac{\text{CFU}}{\text{mg}} \text{ Tablet before exposure to the test medium}} \times 100$$

6.6.7 Survival of CCD optimized Probiotic Tablet after Sequential Incubation in SGF and Simulated Intestinal Fluid (SIF)

The SGF and SIF were prepared according to the United States Pharmacopeia (1995). The SGF was prepared according to U.S. Pharmacopeia (1995) as previously described. The SIF was prepared by dissolving 6.8 g of KH_2PO_4 in 250 ml of water. Then, 77 ml of NaOH (0.2 N) and 500 ml of water, 1.25 g of pancreatin and 3 g of bile salts were added to the solution. Finally, the pH was adjusted at 7.0 with NaOH (0.2 N) or HCl (0.2 N). The SIF was completed with water to obtain 1000 mL. The SIF was sterilized by filtration with a Bottle Top Vacuum Filter (0.2 μm pore size). This sequential incubation analysis was based on the method described by Rao *et al.*, (1989). Probiotic tablets were placed in a tube containing 10ml of sterile SGF (pH 1.5) and incubated at 37° C for 2h with mild agitation (100 rev/min) in a G24 Environmental Incubator Shaker (New Brunswick Scientific Co., USA). After incubation, the tablets were removed and placed in 10ml of sterile SIF (pH 7.0). The tubes were then again incubated at 37° C for 180 min (3h). After incubation, a 1.0-ml aliquot of dissolved tablet was collected and analyzed.

6.6.8 Swelling Study

Tablets were kept in the same conditions as for the sequential transition (from SGF to SIF) test (at 100 rpm and 37° C). For the swelling study, the hydrated tablets were removed from the dissolution medium after 2h in SGF and 3hr in SIF, blotted with tissue paper to eliminate the excess surface water and then weighed. The percentage of water uptake was determined gravimetrically as previously described (Calinescu and Mateescu, 2008).

$$\text{Swelling (\%)} = \frac{W_t}{W_0} \times 100$$

Where, W_t is the weight of the tablets at time t and W_0 is the weight at time 0.

6.6.9 Storage of CCD Optimized Probiotic Tablets

CCD optimized tablets were stored at 4 and 25° C in an amber glass bottle up to 42 days. The viability of the probiotic cells was determined throughout the storage after certain time intervals.

6.6.10 Determination of Viable Cell Counts

The probiotic tablets were dissolved in 10 mL of phosphate buffer solution (pH 7.0, 0.5M) by using a Lab-blender 400 stomacher (Seward Medical, London, UK) at 260 rpm for 1 min. Then the plate count method was used to determine the number of viable bacterial cells. From this homogenate, appropriate serial dilutions were prepared in 0.1% peptone and 100 µl of each dilution was spread on MRS agar plate (Lactobacilli MRS Broth, EMD Chemicals Inc., Germany), which were incubated for 48 h at 37° C under anaerobic conditions using an anaerobic gas pack and anaerobic jars (Anaerogen, Oxoid Limited, Hampshire, England). Bacterial counts were expressed as log CFU/g (free cell, non-compressed) and log CFU/tablet.

6.6.11 Statistical Analysis

To validate the results obtained during different experimental procedure, each analysis was carried out in triplicate in a completely randomized experimental design. An analysis of variance (ANOVA) and multiple comparison tests of Duncan's were used to compare all the results. Differences between means were considered significant when the confidence interval was smaller than 5% ($P \leq 0.05$). The analysis was performed by the PASW Statistics 18 software (IBM Corporation, Somers, NY, USA). The enumeration of probiotic population was independently replicated three times ($n = 3$), with two measurements per replicate.

6.7 Results and Discussion

6.7.1 Data Analysis from CCD

The main objective for CCD design was to develop a probiotic tablet formulation with biopolymers to protect the viability (%) of *L. rhamnosus* during 2h treatment at SGF condition. Variations in tablet weight were due to the differences of the bulk density of different biopolymers (Late and Banga1, 2010) in the formulations. Tablet weight variations could also demonstrate the protection of probiotic viability. **Table 6.2** represents full experimental design with 4 replicates at the center point and the results obtained for the dependent variables (viability, %). An analysis of the variance was carried out to determine the “lack of fit” and the significant effect of the independent variables on the viability (%) of the probiotic in tablet during SGF treatment. The lack of fit test is a measure of the failure of the model to represent data in the experimental region in which points were not included in the regression. Thus the equation of probiotic viability (%) for tablet was:

$$\text{Viability (\%)} = 48.38808 + 0.00196 \times X_1^2 + 0.00179 \times X_2^2 - 0.28254 \times X_3 + 0.00091 X_3^2 - 0.00290 \times X_1 \times X_2 \quad (\text{Eq1})$$

The above equation indicated the quantitative effect of processing variables (X_1 , X_2 , and X_3) and their interactions on the response viability (%). The values of coefficients X_1 to X_3 were associated with the effect of these variables on the response. The regression coefficient, R^2 of the model represents the fraction of variation in the response which could be explained. It is important that this percentage is at least higher than 0.80 (Adjallé *et al.*, 2011). In the current study, the R^2 obtained by the ANOVA method for the viability (%) of probiotic in tablet during SGF treatment was found to be 0.87 (on the basis of Eq-1) and lack-of-fit test (p -value greater than 0.05) indicated that model was fitted adequately to represent the observed data at a 0.87 confidence level. For the viability (%) of *L. rhamnosus* values, statistical analysis of the coefficient of regression (R^2) in the probiotic tablet showed i) quadratic coefficient of the variable X_1^2 produced a statistically significant positive effect ($P \leq 0.003$), ii) quadratic coefficient of the variable X_2^2 produced a statistically significant positive effect ($P \leq 0.005$), iii) linear coefficient of the interaction between variables $X_1 \times X_2$ produced a statistically significant negative effect ($P \leq 0.004$) and iv) linear and quadratic coefficient of the variable X_3 did not produce a statistically significant effect ($P > 0.05$ and $P > 0.10$, respectively).

This suggested that there was a curvature in the response and there were optimal values for these variables. The response surface plot in **Figure 6.1A**) elicited the effect of concentration of alginate (X_1) and pectin (X_2) and their interaction on the viability (%) of *L. rhamnosus* for probiotic tablets. Increasing the concentration of alginate (X_1) or pectin (X_2) (around 170-240 mg) increased the viability of *L. rhamnosus* during 2hr SGF treatment. **Figure 6.1B** represents the response surface plot on the effect of concentration of alginate (X_1) and CNC (X_3) and their interaction on the viability (%) of *L. rhamnosus* for probiotic tablets. It was found that increasing the concentration of alginate with CNC (around 170-240 mg) increased the viability (around 40-80 %). **Figure 6.1C**

exhibits the response surface plot on the effect of concentration of pectin (X_2) and CNC (X_3) and their interaction on the viability (%) of *L. rhamnosus* for probiotic tablet. The results showed that **(Figure 6.1C)** the concentration of pectin and CNC (around 200-240 mg) increased the viability (around 40-60 %) of probiotic tablets. Alginate, a sodium salt, has the ability to form viscous solutions and gels in contact with aqueous media which has been widely used in the pharmaceutical industry. It showed a wide application as a carrier of matrix for controlled release of oral dosage forms (Sriamornsak et al., 2007a). Sriamornsak *et al.* (2007b) also reported that pectin exhibited similar characteristics for the tablet. It is believed that alginate is important to protect the probiotics at gastric pH (1.5) due to its acid gel character below the pK_a of the urinate residues (~ 3.5) (Cook *et al.*, 2011). Combination of alginate and pectin could form more viscous gel at gastric pH which would be a good matrix for the protection of probiotic though the combination of these two matrix elements which are not utilized yet in the pharmaceutical dosage industry. But the combination of alginate and pectin has been found to be a very promising matrix for beverage application as a dietary supplement (Pelkman *et al.*, 2007). In this study, CNC did not improve the viability of probiotics at gastric pH but CNC was found to be important due to its disintegrating properties (Kolakovic *et al.*, 2011). CNC has the property to encourage the disintegration of the tablet at the intestinal of pH-7 to release the bacteria in order to obtain the health benefit. Use of CNC in the pharmaceutical field has not been reported so far. From this design, critical values for alginate, pectin and CNC were 133.65, 163.99 and 155.02 mg/tablet, respectively. According to this design, the critical point demonstrates that at least this quantity of polymer is needed to protect the viability of probiotic. Thus, the tablet was formulated by the combination of these three polymers weight (452.66 mg) which was denoted as CCD. Individual polymers such as alginate (452.66 mg), pectin

(452.66 mg) and CNC (452.66 mg) were also used for the probiotic tablet for the comparison with CCD design.

6.7.2 Survival of CCD optimized Probiotic Tablets after Sequential Incubation in SGF and SIF

The viability (%) of probiotics in tablet formulations during sequential transition through SGF (2h) to SIF (3 h) is presented in **Figure 6.2**. The freeze dried free cell (non-compressed) showed a rapid reduction of bacterial viability, passing from 9.08 log CFU/g to an undetectable level after only 30 min exposure to SGF. This result was expected as *L. rhamnosus* are known to be very sensitive to acidic conditions (Le-Tien *et al.*, 2004). Freeze dried *L. rhamnosus* coated with the CCD optimized tablet formulation (alginate, pectin, CNC, 452.66 mg) exhibited the highest protection of the probiotic during the complete transition through SGF to SIF. The CCD-optimized tablet formulation reduced ($P \leq 0.05$) the viability by 0.08 log (from 8.98 to 8.90 log CFU/tablet) and the viability (%) was 84% after sequential transition from SGF to SIF. On the other hand, freeze dried *L. rhamnosus* coated with alginate, pectin, CNC tablet (453 mg each) failed to show a better protection against the harsh conditions. Results showed that the probiotic viability was reduced ($P \leq 0.05$) by 1.01, 0.77 and 0.73 log (**Table 6.3**) for CNC, alginate and pectin tablet, respectively. The viability (%) for CNC, alginate and pectin probiotic tablets was only 10, 17 and 19%, respectively. Thus combination of different polymer excipients according to CCD improved the viability (%) of the probiotic around 65% compared to the individual addition of the polymer excipients. Calinescu and Mateescu (2008) reported that chitosan coated high amylose starch probiotic tablets (*L. rhamnosus*) showed a viability of 6.5 log after complete transition through SGF to SIF. Poulin *et al.* (2011) reported a viability around 7 log CFU/tablet for β -lactoglobulin probiotic tablet. It has been recommended that to obtain the proper health benefits from probiotic

bacteria, it should be administered at a minimum level of 10^6 - 10^7 CFU/g at point of delivery or be eaten in sufficient amounts to yield a daily intake of 10^8 CFU/g (Chavarri, 2010; Picot and Lacroix, 2004). Thus our study demonstrated that CCD optimized probiotic tablets showed 8.90 log CFU/tablet after complete transition from the SGF to SIF which is supported by the recommended values.

The formation of hydrogel around the cell pellet has been assumed to assure the protection of cell viability. The acidic fluid found in the stomach needs to permeate through the gel layer before reaching the cells. The presence of hydrogel represents a barrier to protect the cell from the acidic fluid. This gastric acid fluid in the stomach contains pepsin, a proteolytic enzyme, which is produced by the auto-catalytic cleavage of its precursor zymogen, pepsinogen, at the low pH of the stomach. Pepsin could break down proteins in the bacterial membrane (Cook *et al.*, 2012). However, intestinal fluid contains bile salts which are the main compound involved in the destruction of lactic acid bacteria. Bile salts cause cellular homeostasis disruptions by the dissociation of lipid bilayer and integral protein of the cell membranes of the probiotics, resulting in leakage of bacterial contents and ultimately cell death (Sahadeva *et al.*, 2011; Iyer and Kailasapathy, 2005). Hence the deleterious effect of pepsin and bile salts could be minimized as the cells are coated with biopolymers. But it was evident that the biopolymers, used individually as alginate, pectin and CNC, were not able to protect the cell due to the influence of the rheology of polymer gel at different pH medium. It was observed that compacted alginate powder formed an insoluble but highly porous, less retarding gel layer when hydrated at lower pH and it showed a soluble and continuous gel layer at the initiation of the higher pH (small intestine) (Chan and Zhang, 2005; Hodsdon *et al.*, 1995) which demonstrated the sudden release of the cells leading to viable cell reduction during the complete transition through the gastrointestinal tract. Sriamornsak

et al., (2007b) reported that pectin showed a fast drug release at SGF due to its high swelling and erosion properties at lower pH. It is well known that CNC has a very high disintegrating property that would release the cell at the SGF (Kolakovic *et al.*, 2011). But it was found that CNC probiotic tablet showed its ability to keep the cell viable after complete transition from SGF to SIF though the tablet did not show the proper shape. This was probably due to the sulfate charge groups of CNC which makes it readily dispersed in water. On the other hand, CNC could form an agglomeration by absorbing gastric fluid containing HCl (Van den Berg *et al.*, 2007; Beck *et al.*, 2012). This properties of CNC could keep the cell viable inside by a protective shield effect during passing through the stomach. Moreover, mixing of CNC with other two biopolymers (alginate and pectin) produced matrices with improved physical properties. The combination of alginate and pectin improved the probiotic tablet properties under acidic environment by shielding effect around the viable cell. When the matrix was exposed to an acidic environment, sodium alginate and pectin remained insoluble acting as a barrier to the diffusion of the cell. But the incorporation of CNC was important to release viable probiotic cells at the intestinal tract. CNC rendered the biopolymers more hydrated and thus showed a continuous release during the whole transition (Howard *et al.*, 1988; Timmins *et al.*, 1997).

6.7.3 Swelling Behaviour of CCD optimized (alginate-pectin-CNC) Tablet

The swelling study was carried out for all the tablets through the complete transition from SGF to SIF. Results were presented in **Figure 6.3**. The swelling behaviour indicated the rate at which the tablet formulations absorbed the liquid from dissolution media and swelled. The changes in weight, characteristic of water uptake and swelling, started from the beginning and continued up to 5hr of experimental measurement (2h in SGF and 3h in SIF). After 2h passing through the SGF medium, the swelling (%) for alginate and pectin was 519 and 497 %, respectively whereas the swelling

(%) for the CCD optimized tablet was 336 %. This result revealed that the CCD optimized tablet reduced ($P \leq 0.05$) the swelling (%) by 35 and 32 % compared to alginate and pectin, respectively. At the entrance of SIF medium (after 1h), the CCD formulation reduced the swelling (222%) that indicated that this formulation started to dissolve in SIF medium. Alginate and pectin also reduced the swelling (289 and 346%) which demonstrated less dissolving or disintegrating compared to the CCD formulation. After 3h passage through the SIF, CCD formulation showed a continuous reduction of swelling behaviour and the swelling (%) was 59 %. However, alginate and pectin exhibited the swelling of 137 and 148% after 3h passing through the SIF. These results demonstrated that CCD increased the swelling by (or dissolution) 57 and 60% compared to alginate and pectin tablet after 3h passing through the SIF.

Under acidic pH conditions, alginate and pectin formed alginic and pectinic acid due to the inter conversion of carboxylate anions (in salt) to free carboxyl groups (in acid), as the concentration of hydrogen ions increases. Also the pka of alginate and pectin is in between 3-4 (Sriamornsak *et al.*, 2007a). Combination of alginate, pectin and CNC in the CCD- ptimized tablet formulation showed a controlled swelling behaviour from SGF to SIF. When alginate and pectin mixed together, gelling occurs by a synergistic reaction, which is referred to as the strong inter chain contact between the protonated “GG-blocks” in alginate and the methoxy groups in pectin (Toft *et al.*, 1986; Liu *et al.*, 2003). In intestinal fluid, alginate and pectin matrices normally start to swell due to the reverse inter conversion of carboxyl group. On the other hand, CNC has the property to increase disintegration of the tablet (Kolakovic *et al.*, 2011) when it is mixed with alginate and pectin. Thus, the novelty of our CCD formulation is the ionic stabilization between alginate and pectin to ensure a two-step protection (through SGF to SIF) of probiotic. Furthermore, CNC addition enhanced the probiotic protection owing to inter conversion nature (Beck *et al.*, 2012) at

two different pH (1.5 and 7). Moreover, the CCD formulation confirmed a gastro protection and stabilization against pancreatic enzyme by modulating the bacterial control release through SGF to SIF.

6.7.4 Storage Stability of *L. rhamnosus*

The stability of probiotics on the basis of cell viability is one of the major challenges for determining the efficiency of pharmaceutical excipients and dosage forms to protect the cells with a long shelf life.

As the CCD optimized probiotic tablet formulation containing alginate (133.65 mg), pectin (163.99 mg) and CNC (155.02 mg) showed the highest protection of *L. rhamnosus* during sequential transition through SGF to SIF, this formulation was selected to check the stability of *L. rhamnosus* during storage. The storage stability was checked at two different temperatures, 25 and 4° C (**Figure 6.4**) to evaluate the viability of the probiotic cell in the tablet formulations.

At 25°C storage condition (**Figure 6.4A**), the CCD-optimized formulation showed an initial viability from 8.98 to 8.55 log CFU/tablet which was significantly ($P \leq 0.05$) reduced by 0.43 log after 42 days of storage. Moreover, the CNC, alginate and pectin tablets exhibited a viability from 8.96 to 6.34, 8.97 to 5.95 and 8.85 to 4.81 log CFU/tablet, respectively. The viability of *L. rhamnosus* was significantly ($P \leq 0.05$) reduced by 2.62, 3.02 and 4.04 log for CNC, alginate and pectin tablet, respectively, after 42 days of storage at 25°C. Free cells (non-compressed) also showed a loss of viability during storage from 9.08 to 4.73 log CFU/g (4.35 log reduction). Thus the CCD optimized tablet formulation improved the probiotic viability 90, 84, 86 and 89 % compared to free cell, CNC, alginate and pectin tablets respectively, up to 42 days of storage.

At 4°C storage conditions (**Figure 6.4B**), the CCD optimized tablet formulation showed a viability from 8.98 to 8.81 log CFU/tablet which was reduced ($P \leq 0.05$) by 0.17 log after 42 days of storage.

The viability of *L. rhamnosus* decreased from 8.96 to 7.02, 8.97 to 6.90 and 8.85 to 5.67 log CFU/tablet for CNC, alginate and pectin tablet, respectively during storage at 4°C. For free cells (non-compressed), the viability was also reduced from 9.08 to 6.97 log CFU/g during storage. Thus, it was found that the CCD-optimized tablet formulation improved the viability by 92, 91, 95 and 92 % compared to free cell, CNC, alginate and pectin tablet respectively, after 42 days of storage at 4°C. Results demonstrated that the CCD optimized probiotic tablets formulation revealed a steady state reduction on cell viability whereas the other individual polymers and free cell (non-compressed) showed ($P \leq 0.05$) rapid decreases in the viability of cell during storage.

Chan and Zhang (2002) reported a mortality of 2 log for *L. acidophilus* after 5 weeks of storage at 25°C in hydroxypropyl cellulose and alginate tablets. Poulin *et al.*, (2011) observed a reduction of 1.5 log CFU/ β -Lactoglobulin tablets of probiotics at room temperature after 3 weeks of storage. But it is interesting to see that our CCD optimized probiotic tablet formulation only reduced viability by 0.43log CFU/tablet of *L. rhamnosus* during 42 days (6 weeks) of storage at 25° C.

Klayraung *et al.*, (2009) reported less than 1 log of reduction for hydroxypropyl methylcellulose phthalate (Hypromellose Phthalate; HPMCP 55)–sodium alginate tablets containing *L. fermentum* 2311 after 6 weeks of storage at 30 °C which is in agreement with our results obtained after same storage period. At 4°C storage conditions, our developed tablet formulation reduced by 0.17 log of cell viability during 42 days of storage. Other reviewers also demonstrated that probiotic cell viability can keep constant during cold storage periods (Stadler and Viernstein, 2003; Maggi *et al.*, 2000). It was indicated that storage temperature had a great impact on cell viability during storage (Gardiner *et al.*, 2000). This means that cold storage is a beneficial condition for maintaining viability over long storage times. Higl *et al.* (2007) reported that temperatures close to above 0 °C reduced the rate of detrimental chemical reactions, such as fatty acid oxidation. But it is also

important to develop formulation for the stabilization of probiotic at room temperature and under pharmaceutical tablet dosage form with different biopolymers, which would be a remarkable development for long term stabilization of probiotic.

6.8 Conclusion

CCD design was used to produce a highly significant statistical model which can adequately describe or predict the optimization of novel probiotic tablets by the combination of alginate, pectin and CNC. According to this mathematical design, it was found that the increasing concentration of alginate showed a profound impact on protecting the viability of probiotics during gastric passage. Pectin also showed a good protection on the viability and its activity improved with the increasing concentration of CNC. A combination of alginate, pectin and CNC (452.66mg) in probiotic tablets gave better stability after complete sequential transition through the gastrointestinal tract. This formulation improved the viability of probiotic by 65% compared to the probiotic tablets made with individual polymers (alginate, pectin and CNC) after passage through the gastrointestinal tract. It is also important to point out that some minor ingredients may be required to improve the processability of the formulation for large-scale production of tablets.

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Table 6.1: Levels of the factor tested in the CCD

Independent variables	Symbol	Level of factors				
		-2	-1	0	1	2
Alginate	X_1 (mg)	0	45	115	195	241.13
Pectin	X_2 (mg)	0	45	115	195	241.13
CNC	X_3 (mg)	0	45	115	195	241.13

Table 6.2: Results of *L. rhamnosus* viability (%) after 2h SGF treatment by CCD design

Standard Runs	Independent Variables			Dependent Variables		
	X ₁ =Alginate (mg)	X ₂ =Pectin (mg)	X ₃ =CNC (mg)	Viability CFU/mg Tablet Before SGF Treatment	Viability CFU/mg Tablet After SGF Treatment	Viability (%), in CFU/mg Tablet
1	45.0000	45.0000	45.0000	3.67×10 ⁸	7.62×10 ⁷	20.78182
2	45.0000	45.0000	195.0000	1.51×10 ⁸	2.58×10 ⁷	17.13654
3	45.0000	195.0000	45.0000	5.76×10 ⁸	3.03×10 ⁷	52.51736
4	45.0000	195.0000	195.0000	5.93×10 ⁸	1.36×10 ⁷	22.83708
5	195.0000	45.0000	45.0000	1.68×10 ⁸	1.21×10 ⁸	72.06538
6	195.0000	45.0000	195.0000	4.95×10 ⁷	3.64×10 ⁷	73.53535
7	195.0000	195.0000	45.0000	1.16×10 ⁸	3.76×10 ⁷	32.41379
8	195.0000	195.0000	195.0000	8.73×10 ⁷	1.45×10 ⁷	16.60305
9	0.0000	115.0000	115.0000	5.80×10 ⁷	2.16×10 ⁷	37.27011
10	241.1345	115.0000	115.0000	1.63×10 ⁸	5.86×10 ⁷	35.87755
11	115.0000	0.0000	115.0000	1.49×10 ⁸	7.30×10 ⁷	48.93855
12	115.0000	241.1345	115.0000	5.45×10 ⁷	9.22×10 ⁶	16.91131
13	115.0000	115.0000	0.0000	1.84×10 ⁸	4.63×10 ⁷	25.18116
14	115.0000	115.0000	241.1345	4.68×10 ⁷	7.23×10 ⁶	15.47237
15(C)	115.0000	115.0000	115.0000	6.63×10 ⁷	7.17×10 ⁶	10.81761
16(C)	115.0000	115.0000	115.0000	1.30×10 ⁸	1.50×10 ⁷	11.54634
17(C)	115.0000	115.0000	115.0000	1.41×10 ⁸	1.80×10 ⁷	12.71976
18(C)	115.0000	115.0000	115.0000	1.50×10 ⁸	1.54×10 ⁷	10.23627

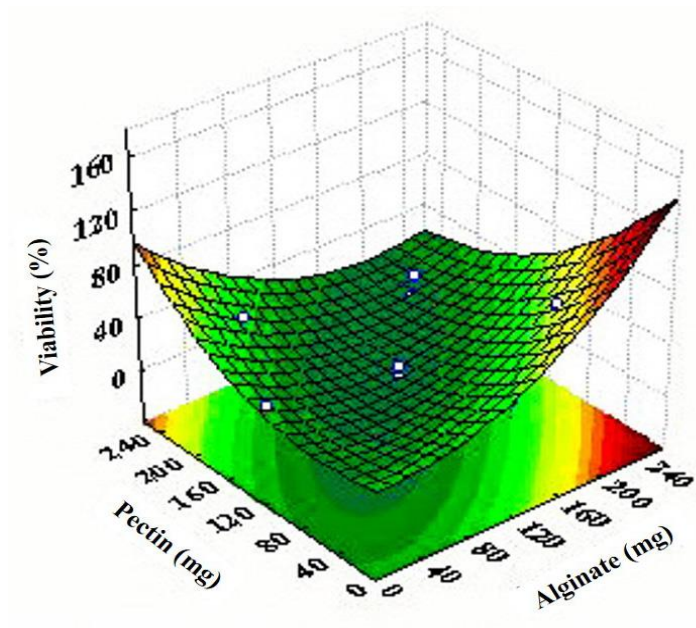


Figure 6.1 (A): Response surface viability (%) of probiotic during SGF treatment obtained by varying alginate (X_1) and pectin (X_2) concentration.

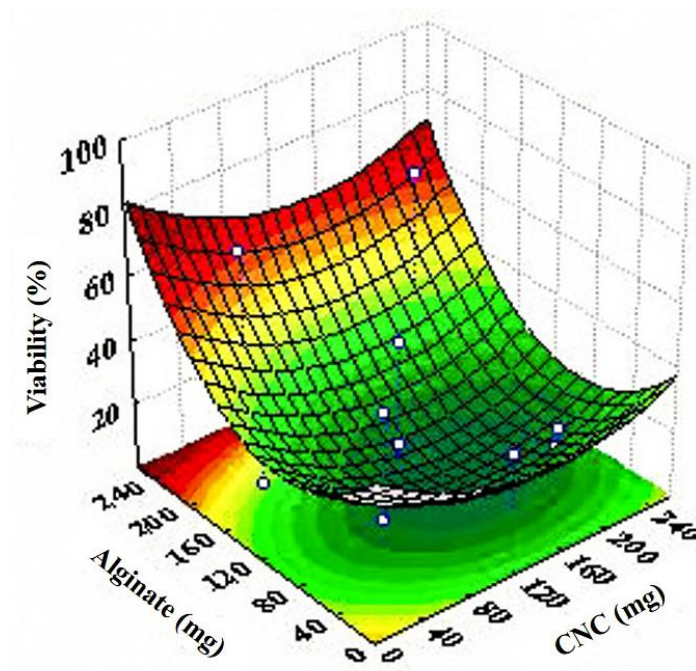


Figure 6.1(B): Response surface viability (%) of probiotic during SGF treatment obtained by varying alginate (X_1) and CNC (X_3) concentration.

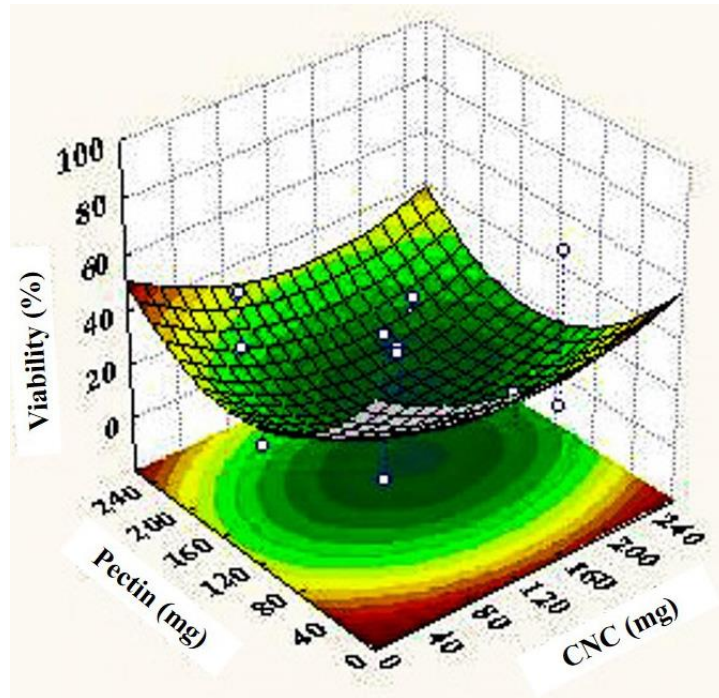


Figure 6.1(C): Response surface viability (%) of probiotic during SGF treatment obtained by varying pectin (X_2) and CNC (X_3) concentration.

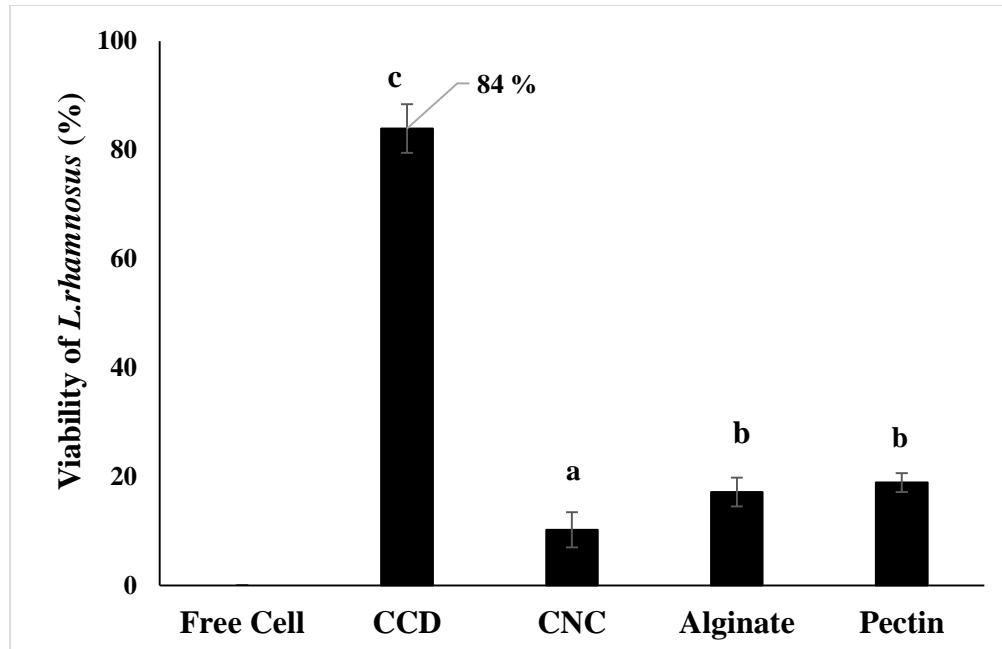


Figure 6.2: Viability (%) of CCD optimized probiotic tablets during sequential transition through SGF (2h) to SIF (3h). Means bearing the same lowercase letter were not significantly different ($P > 0.05$) as determined by multiple comparison test (Duncan).

CCD optimized Tablet: alginate+ pectin + CNC: 133.65+ 163.99+ 155.02 mg (452.66mg)

Table 6.3: Viability of *L.rhamnosus* in CCD optimized Tablet (Log CFU/Tablet) after complete transition through SGF to SIF^{*& **}

Formulation	Before Treatment (Log CFU/mg Tablet)	After Treatment (Log CFU/mg Tablet)
Free Cell	9.08±0.11 ^a	2.00±0.00 ^a
CCD	8.99±0.04 ^{ab}	8.90±0.06 ^d
CNC	8.97±0.13 ^{ab}	7.96±0.05 ^b
Alginate	8.97±0.03 ^{ab}	8.20±0.06 ^c
Pectin	8.86±0.01 ^b	8.13±0.05 ^c

*Means within a column bearing the same letter were not significantly different ($P > 0.05$) as determined by multiple comparison test (Duncan).

**Significant difference ($P \leq 0.05$) was found between before treatment and after treatment after sequential transition through the SGF to SIF samples as determined by the Student t-test.

CCD optimized Tablet: alginate+ pectin + CNC: 133.65+ 163.99+ 155.02 mg (452.66mg)

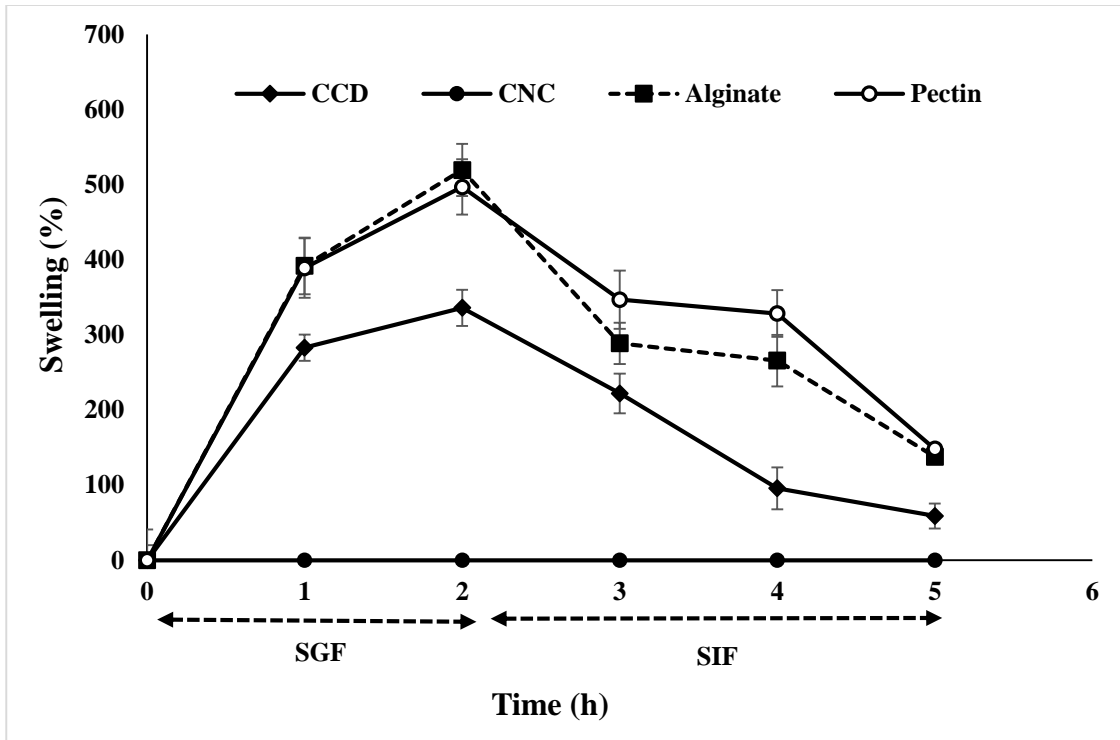


Figure 6.3: Swelling (%) of CCD optimized formulation comparing individual biopolymers (alginate, pectin and CNC) of probiotic tablets during complete transition from SGF to SIF.

CCD optimized Tablet: alginate+ pectin + CNC: 133.65+ 163.99+ 155.02 mg (452.66mg)

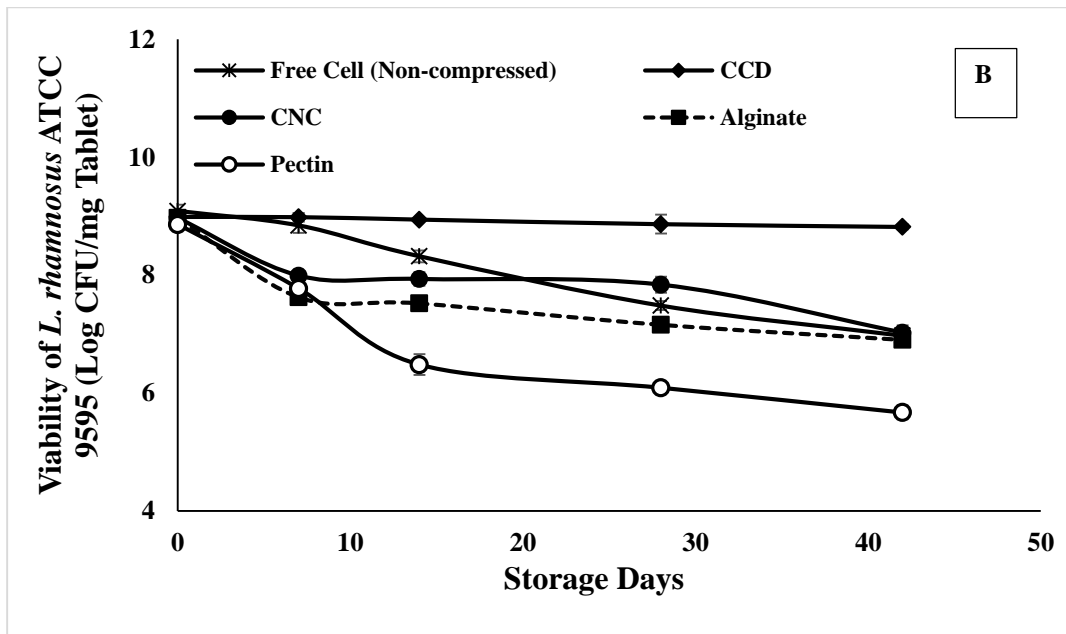
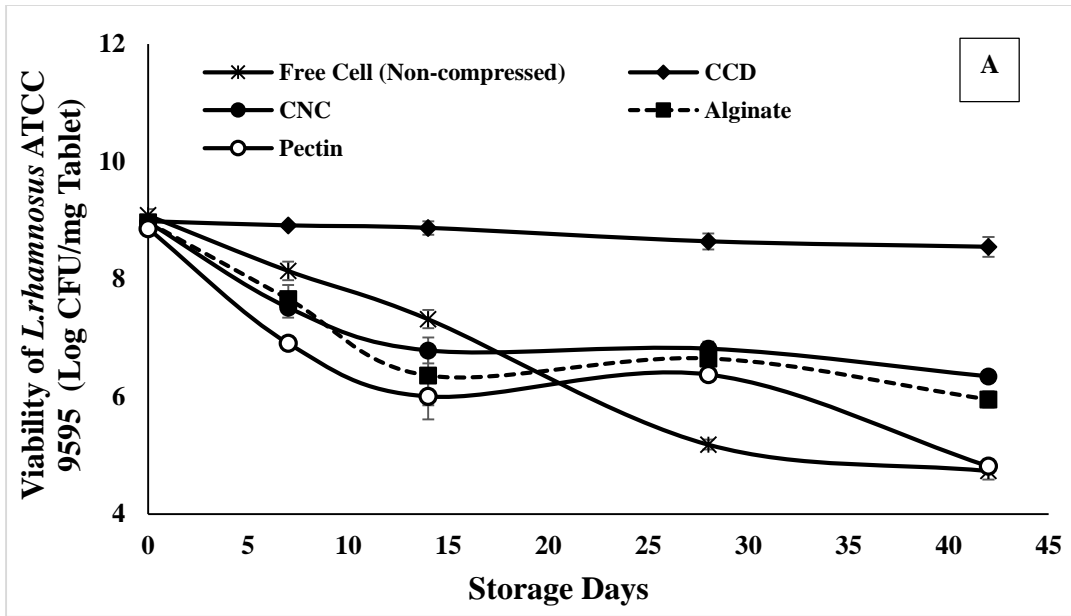


Figure 6.4: Storage stability of *L. rhamnosus* ATCC 9595 containing probiotic tablet A) 25° and B) 4°C.

CCD optimized Tablet: alginate+ pectin + CNC: 133.65+ 163.99+ 155.02 mg (452.66mg)

6.11 General Discussions of the Patent Application

RSM is a very effective method to analyze, predict and model systems that require optimization of multiple factors. The interest of probiotics as remedies for a broad number of gastrointestinal diseases is continuously growing. It is also important to have a good formulation that can keep the recommended viability (6 log CFU/g) of probiotic after ingestion. This reaserch showed that the developed probiotic tablet reduced the viability of probiotic by 0.42 log during storage at 25°C. Also this formulation showed highest viability (84%) of probiotc after complete trasition through SGF to SIF.

Chapter 7

General Discussions/Conclusions and Future Perspectives

This research was carried out for the development of a CNC reinforced biopolymer matrix to be used in food application. CNC possess several favourable characteristics including abundance, renewability, low cost and biodegradability. Thus, it has been extensively used for the microencapsulating matrix development as a reinforcing agent. CNC has been found to be non-toxic and the cytotoxicity results demonstrated that CNC does not have an acute impact on the tested fibroblast cells (3T3 cells). The cell membrane, cell mitochondrial activity and the DNA proliferation remained unchanged which also represented non-toxic characteristics of CNC (Alexandrescu *et al.*, 2013).

Firstly (in chapter 2), the effect of CNC in an alginate matrix was evaluated by characterizing the physicochemical properties. CNC showed its reinforcing properties by improving the mechanical properties of alginate. A mechanically percolating stiff network could form the alginate matrix that supports the mechanical improvement (Dufresne, 2013). CNC also improved the thermal properties of an alginate matrix. Results showed that TGA (thermo-gravimetric analysis) curves were shifted towards higher temperatures after the incorporation of 5 % w/w CNC content that suggests a better thermal stability of the CNC reinforced matrix. This thermal stability also indicated the strong interactions between CNC and the alginate matrix which was also reported by Li *et al.* (2009) and Chang *et al.* (2010) for CNC reinforced chitosan based films and glycerol plasticized starch films, respectively. This developed alginate-CNC matrix was used as a matrix for microencapsulation of antimicrobial compounds (in chapter-3 and 4).

Microencapsulation technology has been found very to be effective to protect antimicrobial compounds from the degradation after contact with food components. From chapter 2, the optimized alginate-CNC (5 % w/w) matrix was used for the microencapsulation of nisin (chapter-3). Nisin was first encapsulated inside in alginate-CNC beads followed by CaCl₂ ionotropic

gelation method (Millette *et al.*, 2007). The beads produced by this method was in millimeter (mm) size which made a distribution problem during RTE meat preparation. Also, as nisin was encapsulated inside the matrix, it was not able to get in contact with the meat and bacteria. In order to overcome these problem, a novel microencapsulation technology was developed that showed a strong antimicrobial activity on RTE meat products against *L. monocytogenes* (a pathogenic bacteria of prime concern due to its high mortality rate). The US Food and Drug Administration (FDA) maintains a policy of “zero-tolerance” for *L. monocytogenes* on RTE meat products. Our developed microencapsulated 63µg/ml of nisin in alginate-CNC microbeads showed an antimicrobial activity during 28 days of storage by *in vitro* study which was demonstrated the complete inhibition of *L. monocytogenes* on RTE meat products for up to 28 days of storage (*in situ* study). Microencapsulated RTE meat products did not change the physicochemical properties of meat during storage. It was also important to evaluate the effect of CNC in alginate matrix for microencapsulation of nisin to enhance its antimicrobial activity. The *in vitro* and *in situ* studies were performed with alginate microbeads without and with 5 % w/w CNC to demonstrate the effect of CNC on nisin activity. It was found that alginate microbeads without and with 5 % w/w CNC did not show any significant ($P \leq 0.05$) difference in the antimicrobial activity of nisin during storage (results were not presented). This could be due to the very low concentration of CNC (5 % w/w) with alginate microbeads.

From chapter 3, the lowest concentration of microencapsulated nisin (16 µg/ml) additionally combined with essential oils (0.025 % w/v or 250 µg/ml) were used to evaluate their synergistic effect with γ -irradiation (chapter 4). Radiation treatment is a process with excellent potential for controlling or eliminating food borne pathogens in food. γ - irradiation is used as a method of food preservation in more than 40 countries, including Belgium, Canada, France, United States and

Netherlands (Turgis *et al.*, 2008). Combined microencapsulation and γ -irradiation has been found to be a novel technology to eliminate *L. monocytogenes* on RTE meat products. Microencapsulated oregano and cinnamon essential oil in combination with nisin showed the highest bacterial radiosensitization of 2.89 and 5, respectively compared to the control. On the other hand, microencapsulated cinnamon EO and nisin in alginate-CNC microbeads showed the lowest growth after γ -irradiation. Oussalah *et al.* (2006) demonstrated the biological processes involving electron transfer by cinnamaldehyde which reacts with vital nitrogen components such as proteins and nucleic acids, thus inhibiting the growth of the microorganisms. Nisin, consisting of 34 amino acids, mainly exhibited its antimicrobial properties against gram positive bacteria such as *L. monocytogenes*. The generally accepted mode of action for nisin on vegetative cells involves the formation of pores in the cytoplasmic membrane of target cells leading to the efflux of essential small cytoplasmic components, such as amino acids, potassium ions and ATP (Prombutara *et al.*, 2012). Additionally, microencapsulation enhanced their antimicrobial activity during storage on RTE meat products. Thus combination of microencapsulated cinnamon and nisin was found to be the most effective antimicrobial on RTE meat products.

For future perspectives, further studies on microencapsulation need to be done in greater detail and it would also be interesting to investigate the mechanism of the action of microencapsulated antimicrobials against pathogenic bacteria. Nisin microencapsulated microbeads could be characterized by fluorescence spectroscopy in order to verify the attachment with CaCl_2 . Drying technology would be the next step to enhance the storage stability of the microcapsules. Sensorial analysis needs to be done for microencapsulated and γ -irradiated antimicrobial containing RTE meat products.

From the literature, it was found that CNC has some characteristics allowing the stabilization of emulsions (Kalashnikova *et al.*, 2012) and it is possible to attach the food grade long chain surfactants with the anionic sulfate group of the CNC (Habibi *et al.*, 2010). A strong interaction was found between negatively H₂SO₄-prepared CNC and the cationic surfactant by way of electrostatic interactions (Xhanari *et al.*, 2010). Nisin could also be attached with CNC by polyelectrolyte complex formation. Thus, in order to maximize the effect of CNC in antimicrobial applications, it is important to first mix the antimicrobials with CNC or modified CNC. It could also be interesting to increase the concentration of CNC in biopolymers to get the effect of CNC. In chapter 5, the effect of CNC on an alginate matrix was studied for probiotic encapsulation. The CNC concentration (5% w/w) in the alginate matrix obtained from chapter 2 was first utilized. But it was found that this concentration of CNC was not enough to protect the viability of the probiotic during freeze drying. This is the reason why the concentration of CNC in alginate microbeads was again optimized by verifying the viability of *L. rhamnosus* ATCC 9595 during freeze drying in chapter 5. It was found that more than 10 % w/w CNC has a great effect to improve the viability of probiotics during freeze drying. Microencapsulation of probiotic is important to protect the bacteria during storage and in the gastro-intestinal tract. It has already been found that alginate alone can not protect the probiotic. Incorporation of CNC in alginate microbeads improved the viability of probiotic during storage (25 and 4° C) and under gastric treatment. Alginate-CNC (13 % w/w) also displayed better mechanical properties. This formulation was further improved by the addition of lecithin. Incorporation of lecithin in alginate-CNC microbeads revealed more protection of probiotic during storage (25 and 4° C) and gastric treatment. But this formulation could not make the probiotic stable after 2 months of storage.

A novel probiotic formulation (CCD tablet) has been developed by central composite design (chapter 6) to overcome the problem (the viability of probiotic was not stable at 25° C) mentioned in chapter 5. CCD tablet showed higher probiotic viability (84%) after sequential transition from SGF to SIF. CNC protected the probiotic in SGF and acted as a disintegrating agent in SIF. The CCD tablet was further compared with two commercial probiotic products and showed a better stability of up to 6 month of storage at room temperature compared to two commercial products. This is the first study regarding the use of CNC in probiotic microencapsulation which will open a new door for the application of CNC in nutraceutical markets.

For future perspectives, the porosity measurement of the freeze dried microbeads needs to be done. SEM characterization should be performed with microencapsulated probiotics in order to get a confirmation about the distribution of the bacteria inside the matrix. The water activity of the CCD tablet needs to be performed during storage. It is also important to characterize the CCD tablet matrix by SEM to check the morphology during swelling. Mucoadhesiveness characterization is one of the most important concerns for the nutraceutical industry which needs to be done for the CCD tablet. To check the viability of probiotics during storage, we used the traditional plate count method. By this method, it would not be possible to obtain the amount of bacteria which were in inactivation state. Hence, it would be promising to adapt the flow cytometry and compare the count with plate count method.

More studies can be done to improve the stability of probiotics during storage. It might be interesting to use spray drying instead of freeze drying. Furthermore, spray drying with controlled temperatures will be allow the reduction of the water activity of the formulation and stabilize the probiotic during storage. As probiotics are temperature and pressure sensitive, it would be

important to verify these spray drying parameters by utilizing mathematical modeling. The next step would be the adhesion study of microencapsulated probiotics with human colon cell lines.

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Annexe-I (Supplementary Data)

Comparative Studies with Commercial Probiotic Nutraceuticals

Tanzina Huq¹, Bernard Riedl², Jean Bouchard³ and Monique Lacroix^{1*}

¹Research Laboratories in Sciences Applied to Food, Canadian Irradiation Centre (CIC),
INRS-Institut Armand-Frappier, Université du Québec, 531 Boulevard des Prairies,
Laval, Québec, H7V 1B7, Canada

²Département des sciences du bois et de la forêt, Faculté de foresterie, géographie et géomatique,
Université Laval, Québec, G1V 0A6, Canada

³FPIinnovations, 570 Boulevard St. Jean, Pointe-Claire, Québec, H9R 3J9, Canada

* **Corresponding Author:** Prof. Monique Lacroix

Telephone: +1-450-687-5010; Fax: +1-450-686-5501; **E-mail:** monique.lacroix@iaf.inrs.ca

Introduction

Probiotics are defined as a live microbial food supplement which beneficially affects the host by improving its intestinal microbial balance. They are recognized to be highly potential for the reduction of harmful bacteria residing in the intestine (Huq *et al.*, 2013). In order to provide health benefits from probiotic bacteria, it has been recommended that they must be present at a minimum level of 10^6 CFU/g of food product or 10^7 CFU/g at point of delivery or be eaten in sufficient amounts to yield a daily intake of 10^8 CFU/g (Chavarri *et al.*, 2010). Recent days, probiotic containing capsules and tablets are developing due to increase the shelf-life of probiotic. Different biopolymers coatings have been used to develop these (capsules and tablets) formulations. In our previous study (chapter 6), probiotic tablet formulation has been developed in our laboratory. Tablet has chosen due to its proper control release in intestine and it can enhance the adhesion and colonization of the probiotic microorganisms to the epithelial mucosa of human host by using the proper kinds of tablet excipients (Maggi *et al.*, 2000). To compare this formulation, we consider two commercial market products to verify the stability during storage and under gastro-intestinal condition.

Materials and Methods

Materials

Sodium alginate (glucuronic acid content ~ 65 - 70%; mannuronic acid content ~5 - 35%) and Pectin (high methyl ester content) was purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). CNC was produced in FP Innovations pilot plant reactor (Pointe-Claire, QC, Canada). Two commercial probiotic capsules (*BioK⁺ Extra Strength probiotic* and *New Root Acidophilus Ultra*) were purchased from Marchés d'Aliments Naturels TAU, Laval, Quebec, Canada. BioK⁺ and Newroot were denoted as commercial-1 and commercial-2, respectively.

Freeze Dried Probiotic Culture Preparation (Biomass)

Lactobacillus rhamnosus was obtained from ATCC 9595 (ATCC, Manassas, VA, USA) and stored at -80°C in Lactobacilli MRS broth (Difco Laboratories, Detroit, MI, U.S.A.) supplied with 10% (v/v) glycerol added. *L. rhamnosus* ATCC 9595 was transferred twice in MRS broth at 37°C . Culture cells were harvested after 24 h by centrifugation (4500g, 10 min at 4°C), washed and resuspended in 0.1% (w/v) peptone water to a final concentration of $\sim 10^9$ - 10^{10} colony-forming units CFU/ml. The harvested probiotic biomass was frozen at -80°C for 2 to 3 h. Then the sample was dried by freeze-dryer (Labconco Freeze Dry Systems, U.S.A.) at -49°C and 0.09 torr pressure for 48 h (2 days) to obtain water activity, $a_w \sim 0.031$. After freeze drying, the concentration of *L. rhamnosus* was again evaluated and it was around $\sim 10^9$ CFU/ml.

Probiotic Tablet Preparation Method

Our developed probiotic tablet formulation was named as CCD (according to chapter 6). CCD Probiotic tablet ($\sim 450\text{mg}$) was made based on alginate (a_w -0.08), pectin (a_w -0.07) and CNC (a_w -0.05). Probiotic tablet was obtained by direct compression of a homogenous mixture of dry powders containing different polymer excipients (alginate, pectin and CNC) and 30 mg of probiotic *L. rhamnosus* bacteria powder (containing $\sim 10^9$ colony-forming units, CFU/mg). The mixture was compressed at 1.2 T/cm^2 using a manual hydraulic Carver press (Wabash, IN, USA) and 10.0 mm cylinder outfits with flat-faced punches (Calinescu and Mateescu *et al.*, 2008).

Viability of Commercial Probiotics and CCD Tablet after Incubation in Simulated Gastric Fluid (SGF)

The SGF was formulated according to United States Pharmacopeia, 1995 and composed of 3.2 g/L of pepsin (approx. 600 units/mg, with one enzyme unit producing a ΔA_{280} of 0.001/min at pH 2.0 and 37°C , measured as trichloroacetic acid-soluble products from hemoglobin as a substrate) and

2.0 g/L NaCl and pH was finally adjusted to 1.5 using HCl (1.0 M) solution. SGF was sterilized by filtration with a Bottle Top Vacuum Filter (0.2 μ m pore size; Nalge Nunc International, NY, USA). Commercial probiotics and CCD Tablet were placed in a tube containing 10mL of sterile SGF (pH 1.5) and incubated at 37° C for different time (20-120 min) interval under mild agitation (100 rev/min) in a G24 Environmental Incubator Shaker (New Brunswick Scientific Co., USA). After incubation, all the formulations were removed and dissolved in phosphate buffer (pH 7, 0.5 M). A 1.0-mL aliquot of dissolved tablets was collected and analyzed.

Release of Commercial Probiotics and CCD Tablet under Simulated Intestinal Fluid (SIF) (Sequential Incubation in SGF and SIF)

The SGF and SIF were prepared according to the United States Pharmacopeia (1995). The SGF was prepared according to U.S. Pharmacopeia (1995) as previously described. The SIF was prepared by dissolving 6.8 g of KH₂PO₄ in 250 mL of water. Then, 77 mL of NaOH (0.2 N) and 500 mL of water, 1.25 g of pancreatin and 3 g of bile salts were added to the solution. Finally, the pH was adjusted at 7.0 with NaOH (0.2 N) or HCl (0.2 N). The SIF was completed with water to obtain 1000 mL. The SIF was sterilized by filtration with a Bottle Top Vacuum Filter (0.2 μ m pore size). This sequential incubation analysis was based on the method described by Rao et al., (1989). Probiotic tablets were placed in a tube containing 10mL of sterile SGF (pH 1.5) and incubated at 37° C for 1hr with mild agitation (100 rev/min) in a G24 Environmental Incubator Shaker (New Brunswick Scientific Co., USA). After incubation, formulations were removed and placed in 10mL of sterile SIF (pH 7.0). The tubes were then again incubated at 37° C for 30, 60, 90 and 120 min under SIF. After incubation, 1.0-mL aliquot of formulations was collected to check the release of probiotic at SIF and analyzed.

Storage of Commercial Probiotics and CCD Tablet

CCD optimized tablets were stored at 4 and 25° C in an amber glass bottle up to 42 days. The viability of the probiotic cells was determined throughout the storage after certain time intervals.

Determination of viable cell counts

All probiotic formulations were dissolved in 10 mL of phosphate buffer solution (pH 7.0, 0.5M) by using a Lab-blender 400 stomacher (Seward Medical, London, UK) at 260 rpm for 1 min. Then the plate count method was used to determine the number of viable bacterial cells. From this homogenate, appropriate serial dilutions were prepared in 0.1% peptone and 100 µl of each dilution was spread on MRS agar plate (Lactobacilli MRS Broth, EMD Chemicals Inc., Germany), which were incubated for 48 h at 37° C under anaerobic conditions using an anaerobic gas pack and anaerobic jars (Anaerogen, Oxoid Limited, Hampshire, England). Bacterial counts were expressed as log CFU/tablet or log CFU/capsule.

Statistical Analysis

To validate the results obtained during different experimental procedure, each analysis was carried out in triplicate in a completely randomized experimental design. An analysis of variance (ANOVA) and multiple comparison tests of Duncan's were used to compare all the results. Differences between means were considered significant when the confidence interval was smaller than 5% ($P \leq 0.05$). The analysis was performed by the PASW Statistics 18 software (IBM Corporation, Somers, NY, USA). The enumeration of probiotic population was independently replicated three times ($n = 3$), with two measurements per replicate.

Results and Discussions

Viability of Commercial Probiotics and CCD Tablet after Different Time Interval under SGF

Table 1 represents the viability of probiotic (log CFU/tablet or capsule) for commercial capsules and CCD tablets. Results showed that after 60 min SGF treatment, commercial-1 and commercial-2 exhibited the viability 8.29 and 6.72 log CFU/capsules, respectively, whereas CCD tablet showed 9.19 log CFU/tablet. Commercial-1 and commercial-2 reduced the viability of probiotic by 1.63 and 2.34 log after 90 min but CCD tablet reduced only by 0.17 log. Similarly, after 180 min SGF treatment, commercial-1 and commercial-2 reduced the viability of probiotic by 2.36 and 3.41 log. It was found that CCD tablet reduced the viability by 0.32 log. These results demonstrated that our developed coating and methodology showed a highest viability of probiotic whereas the commercial formulations drastically reduced their viability.

Release of Commercial Probiotics and CCD Tablet under Simulated Intestinal Fluid (SIF)

Figure 1 shows the viability of probiotic for different formulations after Sequential Treatment (from SGF to SIF). In this study, alginate-pectin tablet was prepared to evaluate the effect of CNC at intestine. Results demonstrated that CNC containing formulation (CCD) showed a controlled release of probiotic at intestine and this formulation (CCD) maintained more than 8 log CFU/tablet throughout the SIF treatment. Whereas without CNC, alginate-pectin formulation showed less than 8 log viability at intestine and the viability was 7.02, 7.04, 6.45 and 6.21 log CFU/tablet after 30, 60, 90 and 120 min SIF treatment, respectively. This results revealed that CNC is important to release (disintegrate) the probiotic at intestine. Commercial-1 showed the viability 6.77 and 6.51 log after 30 and 60 min SIF treatment but after that Commercial-1 could not maintain the recommended (6 log) viability of probiotic at intestine. Commercial-2 did not show the

recommended viability (6 log) at intestine. Thus CCD would be the best formulation amongst all of them.

Storage Stability

Figure 2 and 3 represents the storage stability of commercial capsules and CCD tablets during storage at 25 and 4° C. After 6 month of storage at 25°C, viability of probiotic for commercial-1 and commercial-2 reduced from 9.92 to 4.13 (by 5.79 log) and 9.06 to 6.17 (by 2.89 log) log CFU/capsules, respectively. Whereas CCD formulation reduced the viability of *Lactobacillus rhamnosus* ATCC 9595 from 9.36 to 8.71 (by 0.65 log) log CFU/tablet. Thus, CCD improved the viability 89 and 78% compared to commercial-1 and commercial-2, respectively.

After 6 month of storage at 4°C, viability of probiotic for commercial-1 and commercial-2 reduced from 9.92 to 8.72 (by 1.20 log) and 9.06 to 7.51 (by 1.55 log) log CFU/capsules, respectively. Whereas CCD formulation reduced the viability of *Lactobacillus rhamnosus* ATCC 9595 from 9.36 to 8.78 (by 0.58 log) log CFU/tablet. Results demonstrated that CCD tablet formulation improved the viability 51 and 63 % compared to commercial-1 and commercial-2, respectively.

Conclusion

This work has been done from the continuation of chapter 6 in order to compare the commercial probiotics with our developed formulation. The main importance of this work has found that commercial probiotic formulations do not show better stability during storage and gastric treatment. Whereas our developed formulation showed better stability which demonstrates that it could be good to commercialize probiotic in tablet form. This technology will bring more stable probiotic in market though some more studies need to be done.

Acknowledgement

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Table-1: Viability of Probiotic after Simulated Gastric Fluid (SGF) Treatment*

	Viability of Probiotic (log CFU/tablet or capsule)						
	0 min	20 min	40 min	60 min	80 min	100 min	120 min
Commercial-1	9.92±0.08 ^{Ec}	8.80±0.13 ^{Db}	8.66±0.12 ^{Cc}	8.29±0.11 ^{Bc}	8.27±0.06 ^{Bc}	7.65±0.14 ^{Ac}	7.56±0.10 ^{Ac}
Commercial-2	9.06±0.14 ^{Fa}	7.20±0.12 ^{Ea}	6.80±0.09 ^{Da}	6.72±0.09 ^{Da}	6.40±0.16 ^{Ca}	6.16±0.04 ^{Ba}	5.65±0.09 ^{Aa}
CCD	9.36±0.11 ^{Db}	9.27±0.08 ^{CDd}	9.22±0.06 ^{Cd}	9.19±0.05 ^{BCd}	9.17±0.10 ^{BCd}	9.08±0.13 ^{ABd}	9.04±0.09 ^{Ad}

*Values are means ± standard deviations. Within each row, means with the same uppercase letter are not significantly different ($P > 0.05$). Within each column, means with the same lowercase letter are not significantly different ($P > 0.05$) as determined by multiple comparison test (Duncan).

Commercial-1: *BioK⁺ Extra Strength probiotic*

Commercial-2: *New Root Acidophilus Ultra*

CCD: Alginate (134 mg), Pectin (164 mg), Cellulose Nanocrystal, CNC (155 mg)

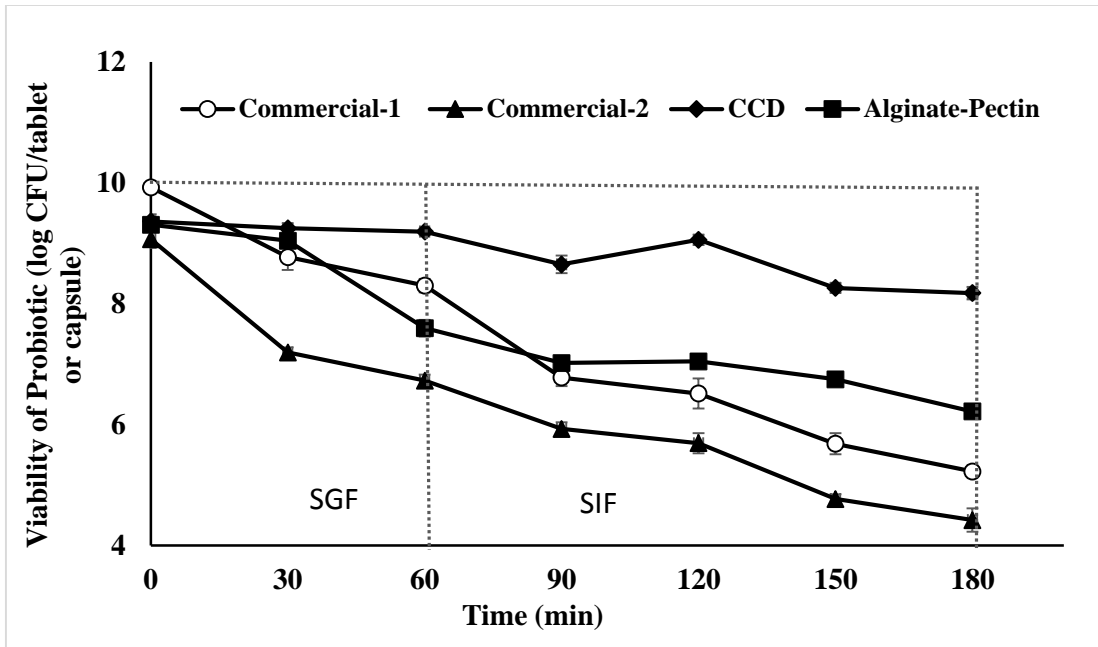


Figure-2: Viability of Probiotic for different formulations after Sequential Treatment (from SGF to SIF)

Commercial-1: *BioK⁺ Extra Strength probiotic*

Commercial-2: *New Root Acidophilus Ultra*

CCD: Alginate (134 mg), Pectin (164 mg), Cellulose Nanocrystal, CNC (155 mg)

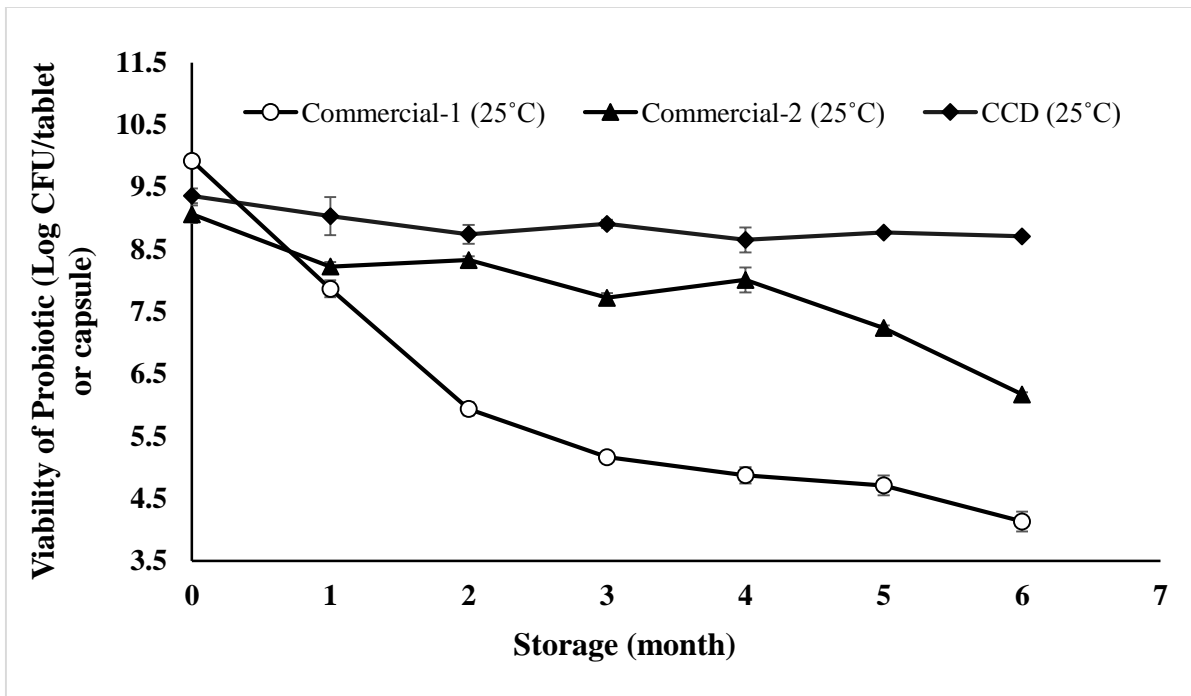


Figure-2: Storage stability of Commercial Probiotic capsules and CCD tablet during storage at 25°C.

Commercial-1: *BioK⁺ Extra Strength probiotic*

Commercial-2: *New Root Acidophilus Ultra*

CCD: Alginate (134 mg), Pectin (164 mg), Cellulose Nanocrystal, CNC (155 mg)

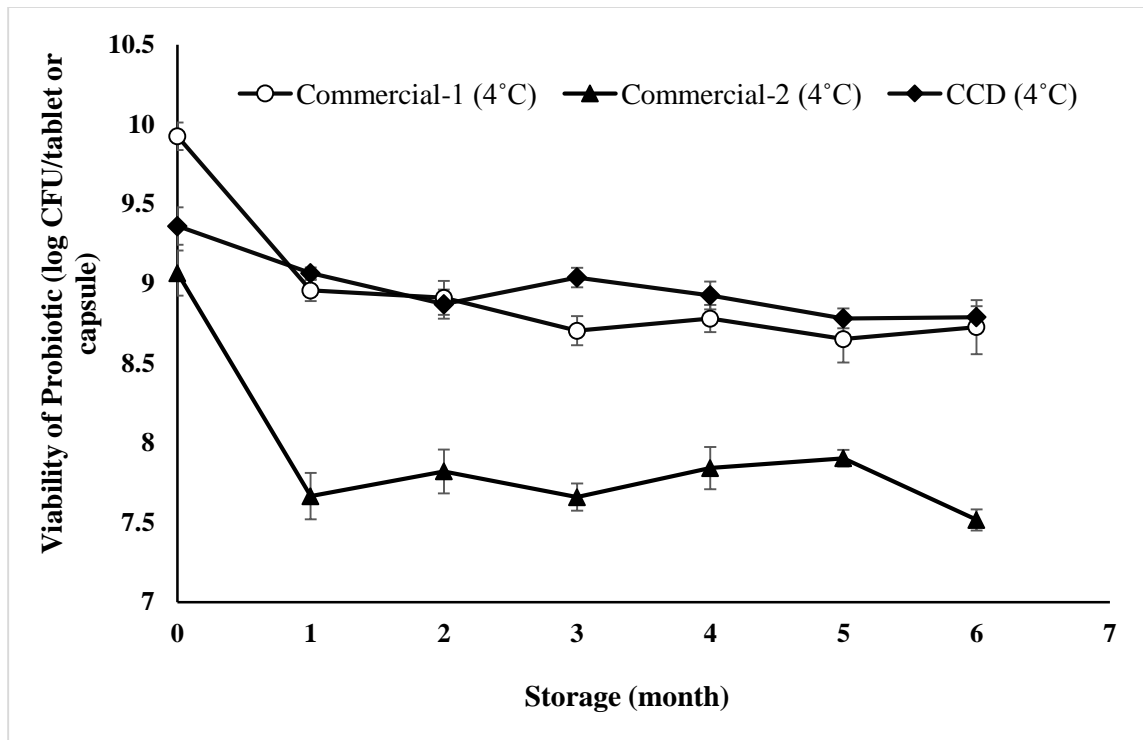


Figure-3: Storage stability of Commercial Probiotic capsules and CCD tablet during storage at 4°C.

Commercial-1: *BioK⁺ Extra Strength probiotic*

Commercial-2: *New Root Acidophilus Ultra*

CCD: Alginate (134 mg), Pectin (164 mg), Cellulose Nanocrystal, CNC (155 mg)

Annexe-II (Supplementary Data)

Microbead Size measurement

Preparation of nisin solution

Different concentration of nisin (0.25, 0.5 and 1% w/v) was prepared by dispersing Niprosin™ powder in 100mL 0.01M CaCl₂ solution and the pH of the nisin-CaCl₂ solution was adjusted to 3-3.5 by diluted lactic acid. Nisin-CaCl₂ solution was stirred overnight for proper mixing. After stirring overnight, the nisin-CaCl₂ solution was centrifuged for 15 min at 3500×g at 4°C to remove the undissolved particles and collected the nisin-CaCl₂ supernatant.

Microencapsulation of nisin

An aqueous suspension was prepared by dissolving 3% (w/v) of alginate in deionized water for 24hr under magnetic stirring. A 1% (w/v) CNC suspension was prepared by dispersing spray dried CNC powder in deionized water under magnetic stirring. Then, the CNC suspension was subjected to ultra-sonication (QSonica Q-500, Misonix, Qsonica, LLC, Newtown, CT, USA) at 1000 J/g of CNC. A 5 % (w/w) CNC from 1 % CNC suspension (according to wt% of alginate) was homogenized using an Ultra-Turrax TP18/1059 homogenizer (Janke and Kunkel, Staufen, Germany) at 23°C and 25000 rpm for 1 min. The different concentrations of nisin-CaCl₂ solutions (16, 31 and 63 µg/ml) were mixed with alginate-CNC suspension (alginate-CNC:nisin-CaCl₂ : 75:25) to form the antimicrobial gel microbeads. The formulations are presented as follows, N1-E 16 µg/ml: micronecapsulated 16 µg/ml nisin, N2-E 31 µg/ml: micronecapsulated 31 µg/ml nisin, N3-E 63 µg/ml: micronecapsulated 63 µg/ml nisin.

Microbead Particle size and Zeta potential analysis

The particle size of alginate-CaCl₂, alginate-CNC-CaCl₂ and microbeads containing 16, 31 and 63 µg/ml of nisin were measured by using the Dynamic Light Scattering Particle Sizer (Malvern

Zetasizer Nano ZS ZEN3600) equipped with a 4.0 MW He/Ne (633 nm) laser beam. To determine the particle size and zeta potential, all the suspensions were diluted 10-fold in water. The measurement was conducted after equilibrating the machine for 30 min at 25°C.

Results and Discussions

Microbead Particle size

The microbead size was determined by dynamic light scattering as shown in **Table-1**. It was found that incorporation of 5wt% CNC in alginate microbead reduced the particle size (Z-average) from 2878 to 751 nm. Addition of different concentration of nisin (16, 31 and 63µg/ml) on alginate microbead gradually increased the size of microbeads. These results demonstrate the evidence of nisin surface adsorption on alginate microbeads. This also highlights the attachment of nisin at the interface of the microbeads and assumes a shrunken conformation to accommodate the particles at nano-level [1].

Table-1: Size of Microbeads

	Particle Size (d nm)
Alginate-CaCl₂	2878 ± 328
Alginate-CNC-CaCl₂	751 ± 34
NE_{CNC}-16µg/ml	760 ± 11
NE_{CNC}-31µg/ml	860 ± 4
NE_{CNC}-63µg/ml	904 ± 18

N1-E 16 µg/ml: micronecapsulated 16 µg/ml nisin in alginate-CNC-CaCl₂ microbeads, N2-E 31 µg/ml: micronecapsulated 31 µg/ml nisin in alginate-CNC-CaCl₂ microbeads, N3-E 63 µg/ml: micronecapsulated 63 µg/ml nisin in alginate-CNC-CaCl₂ microbeads.

Zeta potential

Figure 1 shows the impact of nisin addition in alginate-CNC-CaCl₂ microbeads for the analysis of zeta potential (mV). According to literature, zeta potential is the potential difference between the bulk and slipping plane situated some distance from the particle surface (dependent on the electrical double layer thickness) [2]. The zeta potential value for alginate-CaCl₂ and alginate-CNC-CaCl₂ was -67.2 and -67.7 mV, respectively. Results showed that zeta potential increased after addition of different concentration of nisin in alginate-CNC-CaCl₂ microbeads. The zeta potential was -67.3, -64.5 and -61.5 mV for addition of 16, 31 and 63 µg/ml of nisin in microbeads, respectively. These changes strongly represent the surface adsorption of nisin on microbeads and also demonstrate the stable condition on microbeads in suspension [1].

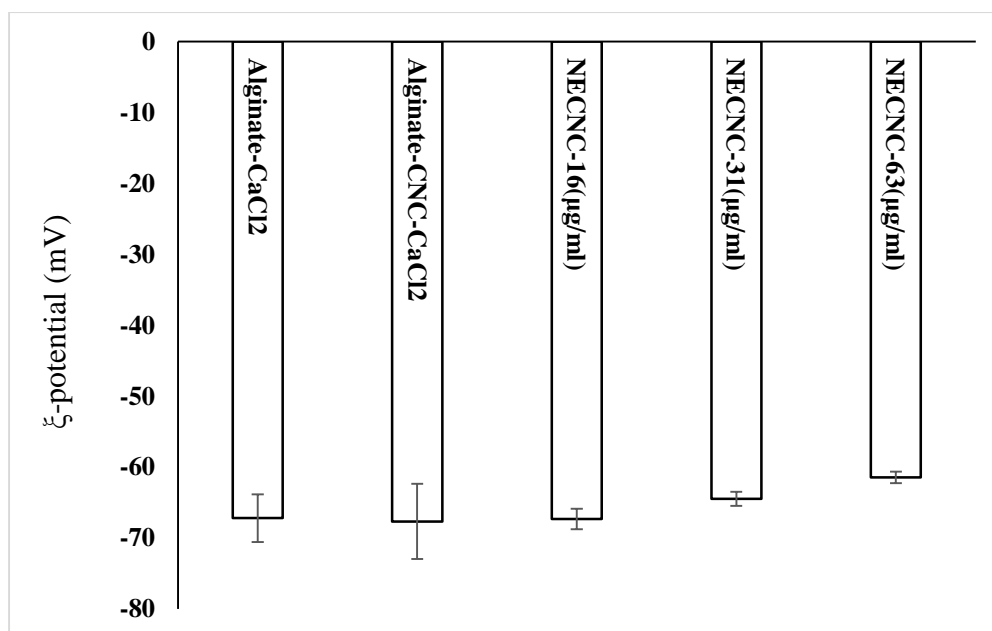


Figure 1: Zeta potential of nisin containing microbeads

N1ECNC- 16 µg/ml: micronecapsulated 16 µg/ml nisin in alginate-CNC-CaCl₂ microbeads,

N2ECNC- 31 µg/ml: micronecapsulated 31 µg/ml nisin in alginate-CNC-CaCl₂ microbeads,

N3ECNC- 63 µg/ml: micronecapsulated 63 µg/ml nisin in alginate-CNC-CaCl₂ microbeads.

If all the particles in suspension exhibit a large positive or negative zeta potential, they will repel each other and there will be no inclination for the particles to come together. But, if the particles exhibit low zeta potential (both positive and negative), then there will be inadequate repulsion to prevent the particles from coming together. The common dividing line between unstable and stable suspensions is taken as +30 or -30mV; particles having zeta potentials beyond these limits are generally considered as stable [3]. Current study revealed that incorporation of CNC in alginate microbead did not change the zeta potential which also represents no aggregation of CNC in alginate matrix.

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Annexe-III (Biography)

List of Publications

1. **Huq, T.**, Riedl, B., Bouchard, J. & Lacroix, M., Microencapsulation of Nisin for Prolonged Efficacy against *Listeria monocytogenes*: *in vitro* and *in situ* studies. Accepted in *Cellulose*, 3rd September, 2014.
2. **Huq, T.**, Vu, K. D., Riedl, B., Bouchard, J. & Lacroix, M., Synergistic Effect of Gamma (γ)-Irradiation and Microencapsulated Antimicrobials against *Listeria monocytogenes* on Ready-to-Eat (RTE) Meat. Accepted in *Food Microbiology*, 12th September, 2014.
3. **Huq, T.**, Riedl, B., Fraschinc, C., Bouchard, J. & Lacroix, M., Development of Cellulose Nanocrystal (CNC) Reinforced Alginate Microcapsule to Protect the Viability of *Lactobacillus rhamnosus* ATCC 9595 During Storage and Gastric Passage. In Preparation for Patent Application.
4. **Huq, T.**, Vu, K. D., Riedl, B., Bouchard, J. & Lacroix, M., Development of Probiotic Tablet Formulation with Cellulose NanoCrystals (CNC) by using Central Composite Design (CCD). In Preparation for Patent Application.
5. Khan, A., **Huq, T.**, Khan, R. A., Riedl, B., & Lacroix, M. (2014). Nanocellulose-Based Composites and Bioactive Agents for Food Packaging. *Critical Reviews in Food Science and Nutrition*, 54(2), 163–174.
6. **Huq, T.**, Khan, A., Khan, R.A., Riedl, B. & Lacroix, M., (2013). Encapsulation of Probiotic Bacteria in Biopolymeric System. *Critical Reviews in Food Science and Nutrition*, 53:909–916.
7. **Huq, T.**, Salmieri, S., Khan, A., Khan, R.A., Le Tien, C., Riedl, B., Fraschinc, C., Bouchard, J., Uribe-Calderon, J., Kamal, M.R. & Lacroix, M., (2012). Nanocrystalline

cellulose (NCC) reinforced alginate based biodegradable nanocomposite film. *Carbohydrate Polymers*, 90, 1757–1763.

8. **Huq, T.**, Khan, A., Dussault, D., Salmieri, S., Khan, R. A., & Lacroix, M. (2012). Effect of gamma radiation on the physico-chemical properties of alginate-based films and beads. *Radiation Physics and Chemistry*, 81(8), 945–948.
9. Khan, A., **Huq, T.**, Khan, R. A., Dussault, D., Salmieri, S., & Lacroix, M. (2012). Effect of gamma radiation on the mechanical and barrier properties of HEMA grafted chitosan-based films. *Radiation Physics and Chemistry*, 81(8), 941–944.

List of Conference Presentations

1. **Huq, T.**, Salmieri, S., Khan, A., Khan, R.A., Le Tien, C., Riedl, B., Fraschinic, C., Bouchard, J., Uribe-Calderon, J., Kamal, M.R. & Lacroix, M., Cellulose Nanocrystals (CNC) reinforced alginate based biodegradable nanocomposite film. International Conference on Bio-based Materials and Composites (ICBMC'14). Montreal, Canada, May 13-16 (*poster presentation*).
2. **Huq, T.**, Dussault, D., Salmieri, S., Le Tien, C., Riedl, B., Bouchard, J. & Lacroix, M., Antimicrobial Edible Microencapsulated Coating Containing Nisin against *Listeria monocytogenes* in Cooked Ham. 58th International Congress of Meat Science and Technology-2012. Montreal, Canada, August 12-17 (*poster presentation, this poster was selected for competition and award among more than 300 posters*).
3. **Huq, T.**, Dussault, D., Salmieri, S., Le Tien, C., Riedl, B., Bouchard, J., Fraschinic, C. & Lacroix, M., Microencapsulation of *Lactobacillus rhamnosus* ATCC 9595 in Alginate-Nanocrystalline Cellulose (NCC) Beads: Effect of Starch and Lecithin. Presented to 2012

TAPPI International Conference on Nanotechnology for Renewable Materials. 4th to 7th June, 2012, Montreal, Quebec, Canada (*oral presentation*).

4. **Hug, T.**, Salmieri, S., Khan, R. A., Riedl, B., Bouchard, J. & Lacroix, M., Effect of Nanocrystalline Cellulose (NCC) on the Survival of *Lactobacillus rhamnosus* ATCC 9595 in Alginate Beads during Freeze Drying and Storage. Nanoquebec Conference, 20th to 21st March, 2012, Montreal, Quebec, Canada (*poster presentation*).
5. **Hug, T.**, Khan, A., Khan, R. A., Dussault, D., Riedl, B. and Lacroix, M., Effect of Gamma Radiation on the Physico-chemical Properties of Alginate Based Films and Beads. International Meeting on Radiation Processing (IMRP 2011), 13th to 16th June, 2011, Montréal, Canada (*poster presentation*).

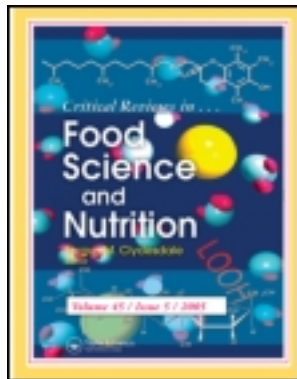
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Tanzina Huq^a, Avik Khan^a, Ruhul A. Khan^a, Bernard Riedl^b & Monique Lacroix^a

^a Research Laboratories in Sciences Applied to Food, Canadian Irradiation Center (CIC), INRS-Institute Armand-Frappier, University of Quebec, 531 Boulevard des Prairies, Laval, Quebec, H7V 1B7, Canada

^b Centre de recherche sur le bois, Faculté de foresterie, de géomatique et de géographie Université Laval, Québec, G1V0A6, Canada

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Encapsulation of Probiotic Bacteria in Biopolymeric System

TANZINA HUQ,¹ AVIK KHAN,¹ RUHUL A. KHAN,¹ BERNARD RIEDL,²
and MONIQUE LACROIX¹

¹Research Laboratories in Sciences Applied to Food, Canadian Irradiation Center (CIC), INRS-Institute Armand-Frappier, University of Quebec, 531 Boulevard des Prairies, Laval, Quebec, H7V 1B7, Canada

²Centre de recherche sur le bois, Faculté de foresterie, de géomatique et de géographie Université Laval, Québec, G1V0A6, Canada

Encapsulation of probiotic bacteria is generally used to enhance the viability during processing, and also for the target delivery in gastrointestinal tract. Probiotics are used with the fermented dairy products, pharmaceutical products, and health supplements. They play a great role in maintaining human health. The survival of these bacteria in the human gastrointestinal system is questionable. In order to protect the viability of the probiotic bacteria, several types of biopolymers such as alginate, chitosan, gelatin, whey protein isolate, cellulose derivatives are used for encapsulation and several methods of encapsulation such as spray drying, extrusion, emulsion have been reported. This review focuses on the method of encapsulation and the use of different biopolymeric system for encapsulation of probiotics.

Keywords Probiotics, biopolymers, prebiotics, encapsulation, SGF, SIF

INTRODUCTION

Probiotics are live microorganisms that transit the gastrointestinal tract and, in doing so, benefit the health of the consumer. They are recognized as very potential bacteria and are also thought that they remove the harmful bacteria from the intestine. Therapeutic benefits have led to an increase in the incorporation of probiotic bacteria such as lactobacilli and bifidobacteria in dairy products, especially yogurts. The efficiency of added probiotic bacteria depends on dose level, and their viability must be maintained throughout storage, products' shelf-life, and they must survive in adverse environment. Hence, viability of probiotic bacteria is of paramount importance in the marketability of probiotic-based food products (Adhikari et al., 2000; Ariful et al., 2010).

Encapsulation of bacterial cells is currently gaining attention to increase viability of probiotic bacteria in acidic products such as yogurt. Encapsulation is a process by which one material or mixture of materials is coated with, or entrapped within, another material or system. The material that is coated or entrapped is

referred to by various names such as core material, payload, actives, fill or internal phase. The material that forms the coating is referred to as the wall material, carrier, membrane, shell or coating. Coating protects the active content from environmental stresses such as acidity, oxygen, and gastric conditions and can be used, for example, to help the content pass through the stomach (Hassan et al., 1996; Dave and Shah, 1997; Godward and Kailasapathy, 2003). Encapsulation segregates the cells from adverse environment, thus potentially reducing cell injury. Encapsulation has been used as a technology that can provide protection against the sensitive probiotic cultures, improving their stability and viability in food products and performing the target delivery in gastrointestinal tract. There is a need for encapsulation of probiotic bacteria to survive human gastric juice in the stomach, where the pH can be as low as 2. The viability of *Bifidobacterium pseudolongum* and *B. longum* in simulated gastric fluid (SGF) environment was improved by encapsulation technology. Encapsulated bacteria showed a higher protection from freezing and freeze drying. A higher stability also showed for Lactic acid bacteria (LAB) during storage of dairy products by using encapsulation (Rao et al., 1989; Lee and Heo, 2000; Shah and Ravula, 2000).

For example, it was reported that encapsulation using calcium-induced alginate–starch polymers, in potassium induced *k*-carrageenan polymers and in whey protein polymers

Address correspondence to Monique Lacroix, Research Laboratories in Sciences Applied to Food, Canadian Irradiation Center (CIC), INRS-Institute Armand-Frappier, University of Quebec, 531 Boulevard des Prairies, Laval, Quebec, H7V 1B7, Canada. E-mail: monique.lacroix@iaf.inrs.ca

have increased the survival and viability of probiotic bacteria in yogurt during storage. The encapsulant materials such as alginate, chitosan, starch, carrageenan, and whey protein are commonly used as food stabilizers in the manufacture of stirred yogurts to prevent syneresis. Alginate is a natural polysaccharide extracted from brown sea weeds and it enhances viscosity and binds water, hence reduces syneresis in stirred yogurts. Divalent cations, such as calcium, bind preferentially to the alginate polymer and, hence, increase viscosity or form gels depending on the concentration. Hi-maize resistant starch has improved thickening and gelling properties and bind water and thicken when added to yogurt hence prevent syneresis and improve textural properties (Siitonen et al., 1990; Sultana et al., 2000).

The aim of this review is to discuss the suitable method of encapsulation for probiotics and also about the encapsulation of probiotic bacteria in biopolymeric system in order to improve the viability and quality of food products during storage and in gastrointestinal tracts.

PROBIOTIC BACTERIAS

People use LAB for more than 4000 years for foods' fermentation. Today, probiotics are also used in a variety of fermented dairy products and their manufacture involves fermentation: microbial process by which lactose is converted into lactic acid. Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) define probiotics as "Live microorganisms (bacteria or yeasts), which when ingested or locally applied in sufficient numbers confer one or more specified demonstrated health benefits for the host" (FAO/WHO, 2001). LABs are the most important probiotic microorganisms typically associated with the human gastrointestinal tract. These bacteria are gram-positive, rod-shaped, non-spore-forming, catalase-negative organisms that are devoid of cytochromes and are of non-aerobic habit but are aero-tolerant, fastidious, acid-tolerant, and strictly fermentative; lactic acid is the major end-product of sugar fermentation. A few of the known LABs that are used as probiotic are *Lactobacillus acidophilus*, *Lactobacillus amylovorus*, *Lactobacillus casei*, *Lactobacillus crispatus*, *Lactobacillus delbrueckii*, *Lactobacillus gasseri*, *Lactobacillus johnsonii*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, etc. (Anal and Singh, 2007). Lactobacilli, as a part of the commensal microbial flora of humans and mammals and main representatives of the probiotic bacteria, might be useful candidates in prevention and treatment of infections caused by multiresistant bacteria due to their ability to modulate the immune responses of the host and to protect the host from pathogens by competitive exclusion (Brachkova et al., 2010; Mohammadi et al., 2011). Other common probiotic microorganisms are the bifidobacteria. Bifidobacteria are also gram-positive and rod-shaped but are strictly anaerobic. These bacteria can grow at pH in the range 4.5–8.5. Bifidobacteria actively ferment carbohydrates, producing mainly acetic acid and lactic acid in a molar ratio

of 3:2 (v/v), but not carbon dioxide, butyric acid or propionic acid. The most recognized species of bifidobacteria that are used as probiotic organisms are *Bifidobacterium adolescentis*, *Bifidobacterium animalis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium infantis*, *Bifidobacterium lactis* and *Bifidobacterium longum*. Other than these bacteria, *Bacillus cereus* var. *toyoi*, *Escherichia coli* strain nissle, *Propionibacterium freudenreichii*, and some types of yeasts, e.g., *Saccharomyces cerevisiae* and *Saccharomyces boulardii* have also been identified as having probiotic effects (Holzapfel et al., 2001).

IMPORTANCE OF PROBIOTICS

Intestinal Tract Health

A number of studies have found probiotic consumption to be useful in the treatment of many types of diarrhoea, including antibiotic-associated diarrhoea in adults, travellers' diarrhoea, and diarrhoeal diseases in young children caused by rotaviruses. The most commonly studied probiotic species in these studies have been *Lactobacillus GG*, *L. casei*, *B. bifidum*, and *S. thermophilus*. Because diarrhoea is a major cause of infant death worldwide and can be incapacitating in adults, the widespread use of probiotics could be an important, non-invasive means to prevent and treat these diseases, particularly in developing countries. Probiotic bacteria have also been shown to preserve intestinal integrity and mediate the effects of inflammatory bowel diseases, irritable bowel syndrome, colitis, and alcoholic liver disease. In addition, LAB may improve intestinal mobility and relieve constipation (Isolauri et al., 1991; Nanji et al., 1994; Pitino et al., 2010).

Nutrient Synthesis and Bioavailability

Fermentation of food with LAB has been shown to increase folic acid content of yogurt, bifidus milk, and kefir, and to increase niacin and riboflavin levels in yogurt, vitamin B₁₂ in cottage cheese, and vitamin B₆ in Cheddar cheese. In addition to nutrient synthesis, probiotics may improve the digestibility of some dietary nutrients such as protein and fat. Short-chain fatty acids such as lactic acid, propionic acid, and butyric acid produced by lactic acid bacteria may help maintain an appropriate pH and protect against pathological changes in the colonic mucosa (Kruis et al., 1997; Chen and Subirade, 2009).

Probiotic Antimicrobial Activity

The importance of probiotics in human nutrition has been gaining recognition in recent years. This study proposed an improved in vitro model for the study of probiotic antimicrobial activity against enteropathogens, by attempting to re-create, in a common culture medium, environmental growth conditions comparable to those present in the small intestine. A preliminary

experiment was carried out in order to find a culture medium able to support both probiotics and pathogens. This was done with the aim of obtaining correct assessment of the interaction under shared growth conditions. Brain Heart Infusion (BHI) medium was selected as the common culture medium and was therefore used in antimicrobial activity assays. The interactions between *Salmonella* 1344 and *Lactobacillus rhamnosus* and *Lactobacillus reuteri* were then assessed at different pH and oxygen availability conditions mimicking the small intestinal environment. *L. rhamnosus* GG ATCC 53103 had the strongest antimicrobial effect, in particular under anaerobic conditions and at lower pH levels. Its antagonistic activity involved both lactic acid and secreted non-lactic acid molecules (Marianelli et al., 2010).

Probiotics for Cancer Prevention

Studies of the effect of probiotic consumption on cancer appear promising. Colorectal cancer (CRC) is the biggest cause of death from cancer in the Western world. Approximately 70% of CRC is associated with environmental factors, probably mainly the diet. The fermented milk containing probiotic cultures can play a protective role against CRC. Interventional studies have shown a shift of intermediate markers of CRC risk in human subjects from a high to low risk pattern after ingestion of fermented milk or probiotics. Animal studies consistently show a reduction in chemically induced colorectal tumor incidence and aberrant crypt formation accompanying probiotic administration. In vitro studies also provide evidence of protection, and permit a better understanding of active compounds involved, and of the mechanisms underlying their anticarcinogenic effects. Probiotics may beneficially modulate several major intestinal functions: detoxification, colonic fermentation, transit, and immune status, which may accompany the development of colon cancer (Saikali et al., 2004). LAB or a soluble compound produced by the bacteria may interact directly with tumour cells in culture and inhibit their growth. LAB significantly reduced the growth and viability of the human colon cancer cell line HT-29 in culture, and dipeptidyl peptidase IV and brush border enzymes were significantly increased, suggesting that these cells may have entered a differentiation process (Baricault et al., 1995; Hirayama and Rafter, 2000).

ENCAPSULATION TECHNOLOGY

Encapsulation can be used for many applications in food industry, including stabilizing the core material, controlling the oxidative reaction, providing sustained or controlled release (both temporal and time-controlled release), masking flavors, colors or odors, extending the shelf-life and protecting components against nutritional loss. A microcapsule consists of a semi-permeable, spherical, thin, and strong membrane surrounding a solid or liquid core, with a diameter varying from a few microns to 1 mm. For enhancing the viability of bacteria, encapsula-

tion facilitates handling of cells and allows a controlled dosage. Food-grade polymers such as alginate, chitosan, carboxymethyl cellulose (CMC), carrageenan, gelatin, and pectin are mainly applied, using various encapsulation technologies (Anal and Singh, 2007).

Extrusion Method

Extrusion method is a simple and cheap method with gentle operations which makes cell injuries minimal and causes relatively high viability of probiotic cells. Biocompatibility and flexibility are some of the other specifications of this method. A hydrocolloid solution is first prepared, probiotics are added, and the solution is dripped through a syringe needle or nozzle. The droplets are allowed to fall into a hardening solution. In this technique, alginate, *k*-carrageenan, *k*-carrageenan plus locust bean gum, xanthan plus gellan, alginate plus corn starch and whey proteins have been used as wall materials for encapsulation of lactobacilli and bifidobacteria. The size of the microcapsules is affected by the nozzle size. The diameter of the obtained alginate beads is also increased as the concentration of sodium alginate increases, but the alginate concentration does not significantly influence the numbers of free cells. A mixture of gellan and xanthan has better technological properties than alginate, *k*-carrageenan, or locust bean gums, but the shape and size of the gellan and xanthan gum capsules have been found to be varying (Rokka and Rantamaki, 2010).

Emulsion Method

Emulsion technique has been successfully applied for the microencapsulation of LAB. In contrary with the extrusion technique, it can be easily scaled up and the diameter of produced beads is considerably smaller (25 μm –2 mm). However, this method requires more cost for performance compared with the extrusion method due to need of using vegetable oil for emulsion formation. In this technique, a small volume of cell/polymer slurry (as a dispersed phase) is added to the large volume of vegetable oil (as a continuous phase) such as soy-, sun flower-, corn-, millet or light paraffin oil. Resulting solution becomes well homogeneous by proper stirring/agitating, till water-in-oil emulsion forms. Emulsifiers can be used for better emulsion formation. Tween 80 at the concentration of 0.2% has been recommended as the best choice. Once W/O emulsion forms, the water soluble polymer becomes insoluble after addition of calcium chloride, by means of cross linking and thus makes gel particles in the oil phase. Smaller particles of the water phase in W/O emulsion will lead to the formation of beads with smaller diameters. Agitation rate of the mixture and type of emulsifier used are also determinable factors from the beads diameter point of view. Using emulsifiers causes formation of beads with smaller diameters, because these components decrease interfacial tension of the water and oil phases. It has been claimed that by applying emulsifiers of tween 80 and lauryl sulphate together,

beads with a range of 25–35 μm in diameter can be produced. In the emulsion technique relevant to alginate, a fat soluble acid such as acetic acid is usually added to the encapsulation mixture. Thereby, pH of alginate solution is reduced to approximately 6.5, at which gelation process of alginate with calcium ions starts. After gel formation, the encapsulated mixture is poured into water to separate the oil phase by decantation. It has been reported that concentration and viscosity of the encapsulation mix before gelation and its agitation rate are the main parameters that control the diameter of the final formed microbeads. It should be reminded that the beads diameter, apart from having a crucial effect on the viability of probiotic cells, their metabolic rate and sensory properties of the final product, also affects distribution and dispersion quality of the microbeads within the product (Krasaekoopt et al., 2003; Picot and Lacroix, 2003).

Drying Method

Drying of the encapsulated mixture in order to produce cell powders/granules can be achieved by different methods. The most important of these methods are freeze drying, spray drying, and fluidized bed drying. Typical survival rates in the spray-drying and freeze drying processes are in the range of 70–85%. Although a survival rate may be acceptable, the prolonged storage stability of the product is often low. The presence of deoxidant and desiccant has been found to improve cell survival. In general, the drying process causes some injuries to the microbeads, release of some cells and reducing viability of the cells. In the freeze drying technique, heat injuries to the cells are minimal compared with other techniques. Also, cryo protectants must be used to inhibit cold injuries to the cells. Spray drying has been recommended for this reason because it is a relatively cheap method and large volumes of solutions can be processed by this technique. However, viability loss of the cells is high due to presence of both dehydration and heating factors, simultaneously. It seems that achieving the best method can be possible by modified techniques of spray drying. This procedure was economic with high ability of maintaining probiotic cells viability. The method consists of coating milk fat droplets containing powder particles of freeze dried cells with polymers of whey proteins, in a condition where emulsifier is used. The size of the starter culture powder particles had a determinable impact on their homogeneous distribution within the oil phase (hydrophobic phase). This size should be bigger than bacterial cells (2–4 μm) and smaller than selected fat droplets (10–50 μm) for achieving appropriate encapsulation. Mentioned size regulations were carried out by the micronization process. It was reported that optimum diameter of fat droplets for the mentioned process was 10–50 μm . Micronization can be done by the size reduction system such as the impact mill, jet mill, mill with agate motor, and ball mill systems. Jet mills form the best systems on both the laboratory and industrial scales. This mill has been used to produce various types of wheat flour, protein powders, and pharmaceutical powders (Dimantov et al., 2003; Picot and Lacroix, 2003).

It was evaluated that the effect of process factors including grind air pressure and feeding rate on the diameter of powder particles and cells viability along with the effect of reducing powder particles size (micronization) on the heat resistance of bacterial cells during the spray-drying process was studied. Micronization was found necessary to reach the homogeneous emulsion system; however, excessive reduction of particles size led to mechanical damage of the cells and considerably decreased their heat resistance during the spray-drying process, especially when high temperatures were used. Therefore, micronization should be carried out with special care and in a particular limit (particularly at high temperatures of spray drying) to avoid mentioned damages. In the research, it was found that dispersing of *Bifidobacterium* spp. fresh cells (unfrozen dried cells) in a suspension of heat-treated whey protein base containing milk fat droplets followed by spray drying of the mixture is a suitable method on the industrial scale with respect to cells viability and economics (Picot and Lacroix, 2004).

ENCAPSULATION OF PROBIOTIC BACTERIA IN DIFFERENT BIO-POLYMERIC SYSTEM

Encapsulation of Probiotics in Alginate Systems

The conventional encapsulation method, with sodium alginate in calcium chloride (CaCl_2), has been used to encapsulate *L. acidophilus* to protect this organism from the harsh acidic conditions in gastric fluid. Studies have shown that calcium-alginate immobilized cell cultures are better protected, shown by an increase in the survival of bacteria under different conditions, than the non-encapsulated state. The results from these studies indicate that the viability of encapsulated bacteria in SGF increases with an increase in capsule size (Anal and Singh, 2007). However, it was reported that very large calcium alginate beads (>1 mm) cause a coarseness of texture in live microbial feed supplements and that small beads of size less than 100 μm do not significantly protect the bacteria in SGF, compared with free cells. These studies indicate that these bacteria should be encapsulated within a particular size range. They tested nine different strains of *Bifidobacterium* spp. for their tolerance to simulated gastrointestinal conditions, and observed some variations among the strains for resistance to gastric fluid (pH 2–3) and bile salts (5 and 10 g/L). Among these strains, only a strain *B. lactis* Bb-12 was found to be resistant to low pH and bile salts. They also encapsulated some of the strains in alginate microspheres to evaluate their resistance properties in gastric fluid and to bile salts. They obtained alginate microspheres (20–70 μm) by emulsifying the mixture of cells and sodium alginate in vegetable oil and subsequently cross-linking with CaCl_2 . Cryo-scanning electron microscopy revealed that these microparticles were densely loaded with probiotic bacteria and were porous. The loaded alginate microparticles remained stable during storage at 4°C in 0.05 M CaCl_2 and in milk (2% fat), sour cream, and yogurt for up to 16 days, and in SGF (pH 2.0)

for 1 h at 37°C. However, the microparticles exposed to low pH did not improve the survival of acid sensitive bifidobacteria. They also showed that *B. bifidum* survived in higher numbers in frozen milk in beads made from alginate than in beads made from *k*-carrageenan (Hansen et al., 2002; Kebary, 1996).

Encapsulation of Probiotics in Proteins and Polysaccharide Mixtures

Gelatin is useful as a thermally reversible gelling agent for encapsulation. Due to its amphoteric nature, it is also an excellent candidate for incorporating with anionic gelforming polysaccharides, such as gellan gum. These hydrocolloids are miscible at pH >6, because they both carry net negative charges and repel one another. However, the net charge of gelatin becomes positive when the pH is adjusted below its isoelectric point and causes a strong interaction with the negatively charged gellan gum. High concentrations of gelatine (24% w/v) were also used to encapsulate *Lactobacillus lactis* by cross-linking with toluene-2, 4-diisocyanate for biomass production (Mortazavian et al., 2007). It was reported that encapsulated *Bifidobacterium* cells in a mixed gel composed of alginate, pectin, and whey proteins. They investigated the protective effects of gel beads without extra membrane and gel beads coated with extra membranes, formed by the conjugation of whey protein and pectin, in simulated gastric pH and bile salt solutions on the survival of free and encapsulated *B. bifidum*. After 1 h of incubation in acidic solution (pH 2.5), the free cell counts decreased by 4.75 log, compared with a decrease of 7.1 log for entrapped cells. The free cells did not survive after 2 h of incubation at pH 2.5, whereas the immobilized cells decreased by about only 2 log. After incubation (1 or 3 h) in 2 and 4% bile salt solutions, the mortality for *B. bifidum* cells in membrane-free gel beads (4–7 log) was greater than that for free cells (2–3 log). However, the counts of cells immobilized in membrane coated gel beads decreased by <2 log. The double membrane coating enhanced the resistance of the cells to acidic conditions and higher bile salt concentrations (Hyndman et al., 1993; Guerin et al., 2003).

Chitosan-Coated Alginate Encapsulate System

Chitin is a homopolymer comprised only of 2-acetamido-2-deoxy- β -D-glucopyranose residues, whereas chitosan is a heteropolymer mainly composed of 2-amino-2-deoxy- β -D-glucopyranose repeating units but still retaining a small amount of 2-acetamido-2-deoxy- β -D-glucopyranose residues. Chitin is the second most abundant organic material on earth after cellulose. Chitosan gel beads and microspheres can be obtained by cross-linking with polyphosphates and sodium alginate (Anal et al., 2003; Anal and Stevens, 2005). Chitosan coating provides stability to alginate microparticles for effective encapsulation of therapeutic live cells. The positively charged amino groups of chitosan and negatively charged carboxylic acid groups of alginate form a membrane on the microparticle surface, which

reduces the leakage of entrapped materials from the particles. Various research works were carried out to investigate the potentiality of a chitosan-coated alginate microparticulate system for increasing the survival and stability of entrapped live probiotic bacterial cells. The survival and stability of probiotic bacteria loaded into chitosan-coated alginate microparticles are largely dependent on the molecular weight of chitosan. *Lactobacillus bulgaricus* KFRI 673-loaded alginate microparticles were coated with chitosans of three different molecular weights to investigate the survival and stability of *Lactobacillus bulgaricus* KFRI 673 in SGF (pH 2.0) and simulated intestinal fluid (SIF) (pH 7.4). Before encapsulation, the authors examined the survival of free *L. bulgaricus* KFRI 673 in SGF of pH 2.0 and in SIF of pH 7.4. In SGF, none of the cells survived after 60 min (Huguet et al, 1996). On the other hand, survival of the *Lactobacillus* strain was fully maintained in SIF over the time period until 120 min, suggesting that *L. bulgaricus* KFRI 673 is pH sensitive and cannot survive in acidic pH conditions. Therefore, encapsulation of the *Lactobacillus* is essential for its survival when given orally. After encapsulation, the survival of *L. bulgaricus* KFRI 673 was investigated for all microparticle batches after sequential incubation in SGF and SIF. The incubation time in SGF was optimized at 0, 30, 90, and 180 min. After then, 180-min incubation was carried out in SIF as for sequential incubation. The microparticles prepared with high molecular weight chitosan provided a higher survival rate (46%) compared with the microparticles made with low molecular weight chitosan (36%). Chitosan-uncoated alginate microparticles showed lower survival (25%) of *L. bulgaricus* KFRI 673. The prepared microparticles stability was also investigated at 4°C and 22°C during a four-week period. Both the free and the encapsulated cells showed similar stabilities at 4°C, whereas high molecular weight chitosan-coated alginate microparticles appreciably improved the *Lactobacillus* stabilities at 22°C compared with free cells and the other respective batches. This was due to the thicker membrane of the microparticles made with high molecular weight chitosan, which protected the encapsulated *Lactobacillus* better than the microparticles made with low and medium molecular weight chitosans and non-encapsulated cells (Lee et al., 2004).

Encapsulation of Probiotics in Cellulose Derivatives

It has been reported that gastric juice resistant tablet formulations of LAB were developed, using hydroxypropylmethylcellulose acetate succinate (HPMCAS) as well as alginates, apple pectin, and Metolose[®] as matrix forming components. To optimize the formulation—using survival rate in acid medium and disintegration time in intestinal fluid as test parameters—tablets were modified with respect to LAB content, amount of applied excipients per tablet, and compaction forces. A decrease of viable cells of not more than one log unit after 2 h of incubation in acid medium was desired, as well as a disintegration time of 1 h in phosphate buffer pH 6.8. It was found that the amount of

HPMCAS in the tablet correlates with gastric juice resistance. As HPMCAS also leads to a decrease of disintegration time in intestinal fluid, slight amounts of this excipient were preferred. The best protective qualities against artificial gastric juice were observed when tablets were prepared from compaction mixtures of LAB, HPMCAS, and sodium alginate (Stadler and Viernstein, 2003). In another report they showed the potential use of compression coating as an alternative method for the encapsulation of probiotic bacteria *Lactobacillus acidophilus* to improve their storage stability. Microbial cell containing powders were first compressed into a pellet, which was then encapsulated with a coating material of a combination of sodium alginate and hydroxypropyl cellulose by further compression. The effect of compression pressure on cell viability was studied. Results showed that compression of the microbial cell containing powders at pressures up to 90 MPa caused little loss of viability of the bacteria. Beyond 90 MPa, the cell viability decreased almost linearly with the compression pressure. Further compression to form a coating did not cause significant reduction in the cell viability. The stability of the encapsulated bacteria using the compression pressures up to 60 MPa was approximately 10 times higher than free cell containing powders and cell pellets after 30 days storage at 25°C (Chan and Zhang, 2002). In another report, they used sodium alginate and hydroxypropyl cellulose as a coating material for encapsulation of Probiotics in acidic medium. Sodium alginate, which can form gels after being hydrated, has been exploited as the prime coating material. Probiotic cell containing powders were first compressed into a pellet, which was then encapsulated with the coating material by further compression. Results indicated significant improvement in survival of encapsulated cells when exposed to acidic media of pH 1.2 and 2. The encapsulated cells showed 10⁴–10⁵-fold increment in cell survival when compared to free cells under the test conditions. The formation of a hydrogel barrier by the compacted sodium alginate layer has shown to retard the permeation of the acidic fluid into the cells. This contributed to the enhanced cell survival. In addition, it could be deduced from in vitro tests that the release of encapsulated cells in the human digestive tract could occur near the end of the ileum and beginning of the colon. The mechanism of cell release is primarily due to the erosion of the alginate gel layer (Chan and Zhang, 2005).

It was reported that cellulose acetate phthalate (CAP) contains ionizable phthalate groups. For this reason, this cellulose derivative polymer is insoluble in acid media at pH 5 and lower but is soluble at pH higher than 6. In addition, CAP is physiologically inert when administered in vivo, and is, therefore, widely used as an enteric coating material for the release of core substances for intestinal targeted delivery systems. It was also reported that the encapsulation of *B. pseudolongum* in CAP used an emulsion technique. Encapsulated bacteria survived in larger numbers (10⁹ CFU/mL) in an acidic environment than non-encapsulated organisms, which did not retain any viability when exposed to a simulated gastric environment for 1 h. Encapsulated *B. lactis* and *L. acidophilus* in CAP polymer used a spray-drying method. This study evaluated the resistance of

encapsulated microorganisms in acid and high bile salt concentrations. Spray-dried microcapsules of CAP containing *B. lactis* and *L. acidophilus* were effective in protecting both these microorganisms when inoculated into media with pH values similar to those in the human stomach. Encapsulated *L. acidophilus* suffered a reduction of only 1 log at pH 1 after 2 h of incubation, and the population of *B. lactis* was reduced by only 1 log immediately after inoculation into a pH 1 medium and between 1 and 2 h after inoculation into a pH 2 medium. After inoculation of the CAP microcapsules loaded with bacteria into bile solution (pH 7), complete dissolution of the powder indicated that both the wall material and the process used in the preparation of the microcapsules were adequate in protecting the bacteria, to pass undamaged through the acidic conditions of the stomach, followed by their rapid liberation in the pH of the intestine (Anal and Singh, 2007).

Encapsulation of Probiotics in Whey Protein Gel particles

Encapsulation of probiotics in whey protein gel particles could offer protection during processing and storage as well as extending the food applications of the bacteria to biscuits, vegetable, and frozen cranberry juice. Whey protein isolate (WPI) has the potential for the encapsulation of *L. rhamnosus* strain. Beads were prepared by extruding the denatured WPI-concentrated bacteria solution and 96% of the probiotic cells were in the whey protein particles. The protein-based technique can provide an alternative for encapsulation with alginate-type gels or spray-coating with fats, the two most widely-used probiotic encapsulation methods. The protein matrix would have different cell release properties than the other encapsulation methods (polymer or fat based). Thus, applications can extend to other foods for protection during processing as well as stability during storage but also in nutraceuticals for protection and cell release in the gastrointestinal tract (Champagne et al., 2006).

Encapsulation of Live Probiotics in Modified Alginate System

Modified alginates were also investigated for encapsulation of live probiotic bacteria to improve their survival in acidic condition. In this regard, succinylated alginate and N-palmitoylaminoethyl alginate were prepared. *Lactobacillus rhamnosus* was microencapsulated into unmodified and modified alginate beads to investigate their acid resistance and viability in acidic condition. To investigate the acid resistance of free cells and encapsulated cells, all the formulations loaded with *Lactobacillus rhamnosus* were incubated in SGF (pH 1.5) for 30 min. For free cells, the initial count was dropped from 1.0 × 10⁸ CFU/ml to an uncountable level after 30 min. Moderate protection was achieved by the unmodified alginate beads loaded with *L. rhamnosus*. Succinylated alginate and succinylated chitosan beads loaded with the probiotic bacteria showed better protection in SGF, with a slight decrease of viability, although

no significant ($P > 0.05$) differences were achieved in protection of encapsulated cells between these two formulations. The best protection in SGF was obtained for N-palmitoylaminoethyl alginate with a slight decrease in bacterial cell viability from 2.5×10^7 to 2.2×10^7 CFU/ml. The minor loss of encapsulated cells from N-palmitoylaminoethyl alginate beads could have occurred from near or on the bead surface. N-Palmitoylaminoethyl alginate beads showed a promising formulation to protect the live bacteria from acidic environment and to improve their survival and stability (Le-Tien et al., 2004).

Effect of Prebiotic for Probiotic Encapsulate System

Adding the prebiotic inulin to yoghurt boosted the growth of probiotic bacteria and when used in a novel double encapsulation, extended the survival rates of the friendly bacteria. The various prebiotic fibers protect the stability and viability of probiotic *Lactobacillus rhamnosus* strains during freeze-drying, storage in freeze-dried form and after formulation into apple juice and chocolate-coated breakfast cereals. The studied prebiotics were: sorbitol, mannitol, lactulose, xylitol, inulin, fructooligosaccharide (FOS), and raffinose (Ann et al., 2007).

Incorporation of Hi-Maize starch (a prebiotic) improved encapsulation of viable bacteria as compared to when the bacteria were encapsulated without starch. Inclusion of glycerol (a cryoprotectant) with alginate mix increased the survival of bacteria when frozen at -20°C . The acidification kinetics of encapsulated bacteria showed that the rate of acid produced was lower than that of free cultures. The encapsulated bacteria, however, did not demonstrate a significant increase in survival when subjected to in vitro high acid and bile salt conditions. A preliminary study was carried out in order to monitor the effects of encapsulation on the survival of *Lactobacillus acidophilus* and *Bifidobacterium* spp. in yogurt over a period of eight weeks. It showed that the survival of encapsulated cultures of *L. acidophilus* and *Bifidobacterium* spp. showed a decline in viable count of about 0.5 log over a period of eight weeks while there was a decline of about 1 log in cultures which were incorporated as free cells in yogurt (Sultana et al., 2000; Vidhyalakshmi et al., 2009). It was reported that prebiotics (FOS or isomaltooligosaccharides) were used as growth promoter (peptide) and sodium alginate as coating materials to encapsulate different probiotics such as *L. acidophilus*, *L. casei*, *B. bifidum*, and *B. longum*. A mixture containing sodium alginate (1% w/v) mixed with peptide (1% w/w) and FOS (3% w/w) as coating materials produced the highest survival in terms of probiotic count (Chen et al., 2005).

CONCLUSION AND FUTURE CHALLENGES

In the food processing industry, encapsulation of probiotics is playing a vital role to protect the viability and enhance the survival of probiotic bacteria against the adverse environmen-

tal conditions. Encapsulated probiotic bacteria can be used in many fermented dairy products, such as yogurt, cheese, cultured cream, and frozen dairy desserts, and for biomass production. In the health food industry, capsules, tablets, suspensions, creams, and powders will be increasingly using encapsulation technology for direct consumption and for external application of probiotics. Encapsulation of probiotic bacteria in foods on an industrial scale faces technological, microbiological, and financial challenges and also questions linked to consumer behaviour. The main challenge in applying encapsulation of probiotics to new foods to meet consumer interests has to do with finding the appropriate encapsulation technique, safe and effective encapsulating materials and potent bacterial strains. Encapsulation is expected to extend the shelf life of probiotics at room temperature in various food matrices, increase their heat resistance, improve their compression and shear stress resistance, and enhance their acid tolerance. Biopolymers are the best effective materials for encapsulation of probiotics. But when only one biopolymer is used for encapsulation, it does not exhibit appropriate effect on encapsulation. Mixture of biopolymers could have the best potential for the encapsulation of probiotics. The future challenge would be the uses of biopolymeric blends for encapsulation of probiotics which will be efficiently protect the probiotics through the gastrointestinal tract where they can interact with specific receptors.

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Effect of gamma radiation on the physico-chemical properties of alginate-based films and beads

Tanzina Huq, Avik Khan, Dominic Dussault, Stephane Salmieri, Ruhul A. Khan, Monique Lacroix*

Research Laboratories in Sciences Applied to Food, Canadian Irradiation Center (CIC), INRS-Institute Armand-Frappier, University of Quebec, 531 Boulevard des Prairies, Laval, Quebec, Canada H7V1B7

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ABSTRACT

Alginate solution (3%, w/v) was prepared using deionized water from its powder. Then the solution was exposed to gamma radiation (0.1–25 kGy). The alginate films were prepared by solution casting. It was found that gamma radiation has strong effect on alginate solution. At low doses, mechanical strength of the alginate films improved but after 5 kGy dose, the strength started to decrease. The mechanism of alginate radiolysis in aqueous solution is discussed. Film formation was not possible from alginate solution at doses > 5 kGy. The mechanical properties such as puncture strength (PS), puncture deformation (PD), viscoelasticity (Y) coefficient of the un-irradiated films were investigated. The values of PS, PD and Y coefficient of the films were 333 N/mm, 3.20 mm and 27%, respectively. Alginate beads were prepared from 3% alginate solution (w/v) by ionotropic gelation method in 5% CaCl₂ solution. The rate of gel swelling improved in irradiated alginate-based beads at low doses (up to 0.5 kGy).

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1. Introduction

Bio-based packaging must serve a number of important functions, including containment and protection of food, maintaining its sensory quality and safety, and communicating information to consumers. A big effort to extend the shelf life and enhance food quality while reducing packaging waste has encouraged the exploration of new bio-based packaging materials, such as edible and biodegradable films from renewable resources. Biodegradable films can be used as a vehicle for the incorporation of food additives such as antioxidants and antimicrobial agents delivering them to the food surfaces where deterioration by microbial growth or oxidation often begins. The demands for high quality foods and opportunities to create new market outlets have contributed to increase the interest in the development of biodegradable packaging. In particular, the application of biodegradable edible films as effective barriers against gas, moisture and liquid migration has appeared to be the appropriate way for prolongation of the shelf-life of ready-to-eat food and for the increase of its quality. Films can be produced from natural polymers, such as polysaccharides, lipids and/or proteins, and are perfectly biodegradable and safe to the environment (Sorrentino et al., 2007; Ciesla et al., 2006; Silva et al., 2009; Zactiti and Kieckbusch, 2006).

Alginate is widely used in food, pharmaceutical and bioengineering industries for its gel- and film-forming properties. Alginate, a linear heteropolysaccharide of D-mannuronic and L-guluronic acid, is found in the cell walls and intercellular spaces of brown algae. Alginate is made up of arranged regions composed solely of D-mannuronic acid and L-guluronic acid, referred to as M-blocks and G-blocks, and regions where the two units alternate. Both the ratio of mannuronic/guluronic acid and the structure of the polymer determine the solution properties of the alginate (Lee et al., 2003; Khan et al., 2010; Simpson et al., 2004; Prakash and Jones, 2005). Alginate forms a thermally stable and biocompatible hydrogel in the presence of di- or trivalent cations. In addition, alginate beads can be easily produced by dropping an alginate solution in a calcium chloride bath (Chana et al., 2010).

Gamma irradiation has been found to be effective for degradation of carbohydrates such as alginate, cellulose, starch, chitosan and pectin. Cleavage of the molecular chain is ascribed to decaying processes of free radicals generated at the primary stage of gamma irradiation. Due to macro-radical formation and their further reactions, degradation methods are accompanied of various extents with changes in chemical composition and primary structure of the polysaccharides (Kim et al., 2008; Choi et al., 2002).

Gamma irradiation is also used for the biological sterilization of materials that can be subsequently used for manufacturing biomedical products. In addition, ionizing radiation leads to the degradation of polysaccharides such as alginate by the cleavage of the glycosidic bonds. The basic advantages of degradation of

* Corresponding author. Tel.: +1 450 687 5010; fax: +1 450 686 5501.

E-mail address: monique.lacroix@iaf.inrs.ca (M. Lacroix).

polymers by radiation include the ability to promote changes reproducibly and quantitatively, without the introduction of chemical reagents and without the need for special equipments/setup to control for temperature, environment and additives. Therefore, this technology is simpler and more environment-friendly than conventional methods (Byun et al., 2008). The objective of this experiment was to determine the effect of gamma radiation on the physico-chemical properties of alginate-based films and beads.

2. Materials and methods

2.1. Materials

Sodium alginate (guluronic acid content ~65–70% and manuronic acid content ~5–35%) and calcium chloride (granules) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

2.2. Irradiation

An aqueous suspension containing 3% alginate (w/v) was prepared and then irradiated from 0.1 to 25 kGy. Irradiation of the 3% alginate solution (w/v) was conducted with γ -rays generated from ^{60}Co source at room temperature, at a dose rate of 17.878 kGy/h (0.3578 kGy/min) in an Underwater Calibrator-15A Research Irradiator (Nordion Inc., Kanata, ON, Canada).

2.3. Preparation of alginate-based beads and films

The alginate-based films and beads were prepared from unirradiated and irradiated alginate suspension. Films were cast by applying 10 mL of the film-forming suspension onto petri dishes ($100 \times 15 \text{ mm}^2$; VWR International, Ville Mont-Royal, QC, Canada) and allowed to dry for 24 h at room temperature and 35% relative humidity (RH). Dried water-soluble films were peeled off manually using a spatula and stored in polyethylene bags prior to characterization. The beads were produced by dropping through a 20-gage needle (0.9 mm in diameter and 1.5 in. in length) from a 10 mL plastic syringe into a beaker containing CaCl_2 solution (5% w/v), under gentle stirring at room temperature. The formed beads were then allowed to harden for 30 min and then rinsed with distilled water (Salmieri and Lacroix, 2006).

2.4. Film and bead thickness

Thickness of the films (thickness ~25 μm) and 10 beads (2–3.5 mm in wet condition and 0.6–2 mm in dry condition) was measured using a Mitutoyo digimatic Indicator (Type ID-110E; Mitutoyo Manufacturing Co. Ltd., Tokyo, Japan) with a resolution of 0.001 mm, at five random positions around the film and bead, by slowly reducing the micrometer gap until the first indication of contact.

2.5. Puncture strength (PS) and puncture deformation (PD)

Mechanical properties were carried out according to an ASTM (1991) D882-91 procedure. Puncture strength (PS) and puncture deformation (PD) measurements were carried out using a Stevens-LFRA texture analyzer (model TA-1000; Texture Technologies Corp., Scarsdale, NY). The film samples were equilibrated in a dessicator containing a saturated sodium bromide solution ensuring 56% RH at room temperature (21 °C) for at least 24 h. Films were then fixed between two perforated Plexiglass[®] plates (3.2 cm in diameter), and the holder was held tightly with two

screws. A cylindrical probe (2 mm in diameter) was moved perpendicularly to the film surface at a constant speed (1 mm/s) until it passed through the film. Strength and deformation values at the puncture point were used to calculate the hardness and deformation capacity of the film. To avoid any variation related to the film thickness, the PS values were divided by the thickness of the films. PS was calculated using the equation $\text{PS (N/mm)} = (9.81 \times F)/x$ where F is the force, x is the film thickness and 9.81 is the gravitational acceleration. The PD of the films was calculated from the PS curve, using the distance recorded between the time of first probe/film contact and the time of puncture point.

2.6. Viscoelasticity coefficient (Y)

Viscoelastic properties were evaluated using relaxation curves. The same puncture test procedure described above was used, but the probe is stopped to 3 mm after film contact and maintained for 1 min. The relaxation coefficient Y is calculated using the equation $Y (\%) = [(F_i - F_f)/F_i] \times 100$ where F_i is the initial recorded value and F_f the second value measured after 1 min of relaxation. A low relaxation coefficient ($Y \rightarrow 0\%$) indicates high film elasticity, whereas a high coefficient ($Y \rightarrow 100\%$) indicates high film plasticity related to a more rigid and easily distorted material.

2.7. Rate of gel swelling

A water uptake apparatus was designed to study the water absorption properties of beads and consequently to determine the rate of gel swelling. Beads were dried at 40 °C for 24 h in a drying oven and placed in a 5 mL graduated cylinder (0.2 mL subdivision). Water penetration into beads was measured as a function of time (Han et al., 2008). The water uptake is expressed in the rate of gel swelling (percent weight increase).

2.8. Statistical analysis

To validate the results obtained during different experimental procedures, each analysis was carried out in triplicate in a completely randomized experimental design. An analysis of variance (ANOVA) and multiple comparison tests of Duncan's were used to compare all the results. Differences between means are considered significant when the confidence interval is smaller than 5% ($p \leq 0.05$). The analysis was performed by the PASW Statistics 18 software (IBM Corporation, Somers, NY, USA).

3. Results and discussion

3.1. Effect of gamma irradiation on the PS and PD of films

The PS and PD results of the un-irradiated and irradiated alginate-based films are presented in Figs. 1 and 2. The PS and PD values of the un-irradiated alginate-based films (control) were found to be 333 N/mm and 3.2 mm, respectively. It was found that from 0.1 to 0.5 kGy, the PS values increased with the increase in gamma radiation dose. At doses of 0.1 and 0.5 kGy, the PS values of the irradiated films were 365 and 375 N/mm, which were about 10% and 13% higher, respectively, compared to un-irradiated alginate-based films. At doses higher than 0.5 kGy, the increase in irradiation dose decreased the PS values of the films significantly ($p \leq 0.05$). At 1 and 5 kGy, the PS values of the films were 356 and 313 N/mm, respectively. The PD values increased with the increase in the dose of gamma radiation and results are shown in Fig. 2. At doses of 0.1 and 0.5 kGy, the PD values were 4.6 and 4.8 mm, respectively, which represented an increase in PD of about 44% and 50%, respectively, compared to un-irradiated

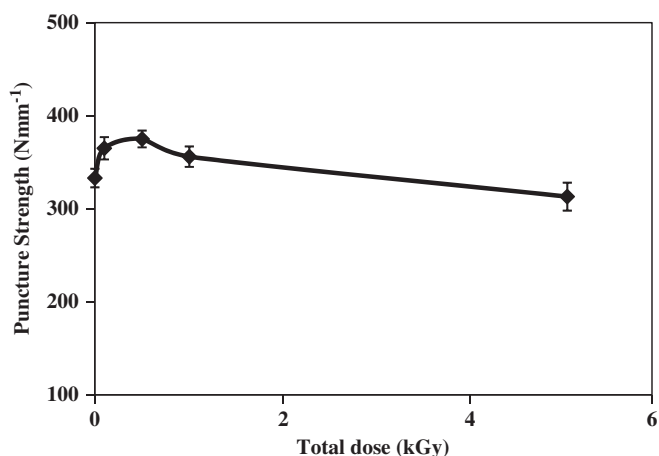


Fig. 1. Effect of gamma irradiation on PS of alginate-based films.

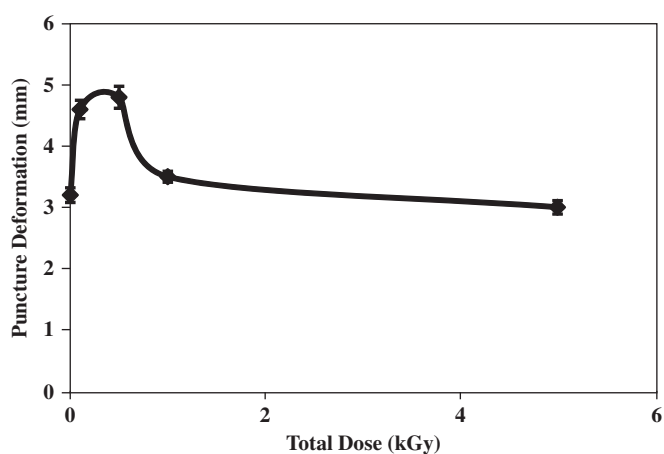


Fig. 2. Effect of gamma irradiation on PD of alginate-based films.

alginate films. At doses higher than 1 kGy, the PD values decreased significantly ($p \leq 0.05$). On the other hand, viscoelasticity (Y coefficient) of irradiated films was found to be almost similar to the un-irradiated alginate-based films. So, it was found that at lower irradiation doses (0.1–0.5 kGy), the PS and the PD values increased significantly ($p \leq 0.05$). The increase in mechanical properties of alginate-based films at low irradiation doses (0.1–0.5 kGy) may be attributed to the generation of free radicals and cross-linking of alginate molecules. However, at doses higher than 1 kGy, both PS and PD values decreased significantly ($p \leq 0.05$). The decrease in PS and PD values may be due to the chain scission when alginate is exposed to gamma irradiation. Alginate, which is a polysaccharide, generally undergoes degradation by the breaking of the glycosidic linkage under higher dose of gamma irradiation. It is also reported in the literature that cellulose and alginate molecules also form free radicals in a similar nature when exposed to gamma radiation (Gul-E-Noor et al., 2009).

3.2. Effect of gamma irradiation on the rate of gel swelling

Effect of gamma irradiation on the rate of gel swelling of alginate-based beads prepared from irradiated alginate solution is presented in Fig. 3. The values of rate of gel swelling improved in irradiated alginate-based beads at doses up to 0.5 kGy. At 0.1 and 0.5 kGy, the rate of gel swelling decreased 18% and 21%, respectively, as compared to the un-irradiated alginate-based beads. At doses higher than 0.5 kGy, the rate of gel swelling increased. At 1 and 5 kGy, the rate

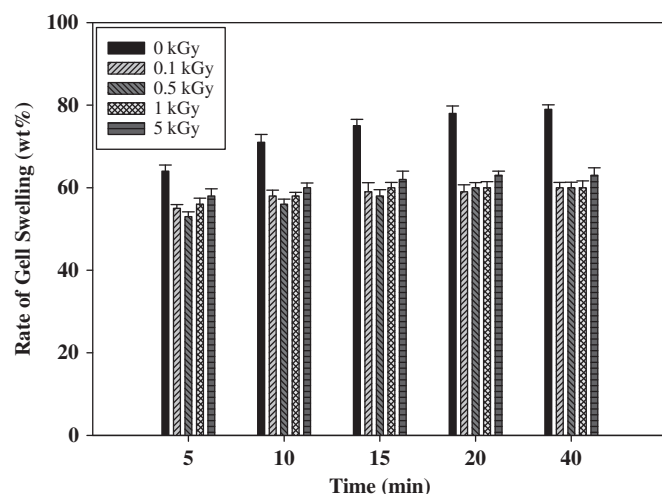
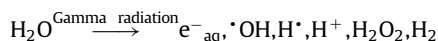


Fig. 3. Effect of gamma irradiation on rate of gel swelling properties of alginate-based beads.

of gel swelling found 58% and 60%, respectively, which was significantly ($p \leq 0.05$) lower than the un-irradiated alginate-based bead.

3.3. Mechanism of radiolysis of alginate in aqueous solution

It is mentioned above that gamma radiation has a strong effect on alginate solution. At low doses, mechanical strength of the prepared films improved but after 5 kGy dose, the strength started to decrease. Here, 3% alginate solution was used. So, water has a great influence on the radiolysis of alginate. The mechanism of alginate radiolysis in aqueous solution is discussed below. Due to exposure of gamma radiation, firstly hydroxyl radicals have been generated by radiolysis of water (Von Sonntag, 1987). The radicals generated by water molecules under the effect of gamma radiation are as follows:



Thus the alginate-derived radicals would be formed almost solely by attack of OH radicals resulting from radiolysis of water. The $\text{H}\cdot$ and $\cdot\text{OH}$ radicals formed by radiolysis of water accelerated the molecular chain scission of alginate. Reaction between the above free radicals and alginate molecules leads to rapid degradation of alginate in aqueous solution. The free radicals formed by radiolysis of water are effective even in enhancing crosslinking. Then, H-atoms abstraction from various C-atoms by OH radicals might be occurred. Thus, in fact radicals located at all carbon atoms (except the carboxylic carbon) will be formed. Since, oxygen was present in the system; the initially formed alkyl radicals would rapidly react with oxygen to form peroxy radicals. Their transformations could lead to chain scission, oxidation with the formation of carbonyl groups, etc. (Von Sonntag et al., 1999). According to Nagasawa et al. (2000), probably hydrogen abstracted indirectly by hydroxyl radical or surrounding macroradical and as a result, a double bond formation could lead to stabilization of alginate radicals. It is reported (Wasikiewicz et al., 2005) that water radiolysis is the main effect of g-irradiation of diluted aqueous solutions. It results in the formation of transient products, which then react with the solute. In the case of diluted aqueous solutions of polycarbohydrates, formed hydroxyl radical and hydrogen atoms are able to abstract hydrogen atoms from the polymer. Thus, macroradicals are formed. Subsequent reactions of macroradicals can be chain scission, hydrogen transfer, inter- and intramolecular recombination and finally disproportionation of macroradicals. Effect of chain scission can be followed by a

decrease in the molecular weight of the polymer. Degradation rate increases with the decrease in the polymer concentration. Mainly, it is caused by the enhanced OH radical mobility rising with dilution of the solution, due to reduced viscosity. Moreover, in diluted solutions the distance between two radicals located on neighboring polymer chains becomes larger. This significantly decreases their recombination possibility, which could give an effect opposite to degradation, i.e., the crosslinking reaction. In the presence of oxygen, the alginate derived radicals are converted into the corresponding peroxy radicals. Due to their long lifetimes, these peroxy radicals can also undergo H-abstraction reactions. This induces chain reaction in this system. According to other researchers (Charlesby, 1958; Mollah et al., 2009; Charlesby and Swallow, 1959; Dole et al., 1959) when natural polymeric materials are subjected to high-energy radiation (gamma), radicals are produced on the main chain by hydrogen and hydroxyl abstraction. Gamma radiation also ruptures some carbon–carbon bonds and produces radicals. Chain scission may also take place to form other radicals. The ionizing radiation produces three types of reactive species in polymer. These are ionic, radical and peroxide. The peroxide species are produced when polymers are irradiated in the presence of oxygen (Charlesby, 1958; Charlesby and Swallow, 1959; Dole et al., 1959). Peroxide reacts with polymers and produces polymer diperoxides (POOP) and hydroperoxides (POOH) by a radical chain reaction process. The reactions occurs in three steps: activation, propagation and termination. The effect of high-energy radiation on polymers (such alginate) produced ionization and excitation; as a result some free radicals are produced. The polymers may undergo cleavage or scission (i.e., the polymer molecules may be broken into smaller fragment). Subsequent rupture of chemical bonds yields fragments of the large polymer molecules. The free radicals thus produced may react to change the structure of the polymer and also the physical properties of the materials. It also may undergo cross-linking (i.e., the molecules may be linked together into large molecules) (Saheb and Jog, 1999; Valdez-Gonzalez et al., 1999). Gamma irradiation also affects the polymeric structure and can produce active site. Gamma irradiation of alginate may result in cross-linking, which produces higher mechanical properties up to a certain dose. Active sites inside the polymer might be produced by the application of gamma radiation. This may be the reason behind the variation (increase and decrease) in the mechanical properties of the prepared alginate films at the exposure of gamma radiation (low and high doses).

4. Conclusion

This study was carried out in order to investigate the effect of gamma irradiation on the physico-chemical and swelling properties of alginate-based films and beads. From this study, it is clear that low gamma irradiation doses (0.1–0.5 kGy) allowed the improvement not only in the mechanical properties but also in the swelling properties of alginate-based films and beads. However, at doses higher than 0.5 kGy, the mechanical properties decreased. The optimum gamma irradiation dose was found to be 0.5 kGy. Hence, low doses of gamma irradiation can conveniently improve the mechanical and swelling properties of alginate-based films and beads.

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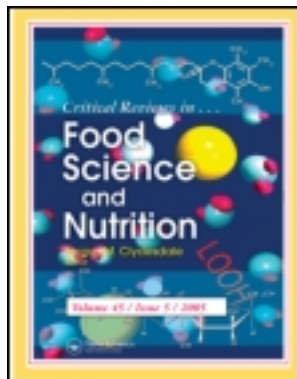
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Avik Khan^a, Tanzina Huq^a, Ruhul A. Khan^a, Bernard Riedl^b & Monique Lacroix^a

^a Research Laboratories in Sciences Applied to Food, Canadian Irradiation Center (CIC), INRS-Institute Armand-Frappier, University of Quebec, 531 Boulevard des Prairies, Laval, Quebec, H7V 1B7, Canada

^b Centre de recherche sur le bois, Faculté de foresterie, de géomatique et de géographie, Université Laval, Quebec, G1V 0A6, Canada

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Nanocellulose-Based Composites and Bioactive Agents for Food Packaging

AVIK KHAN,¹ TANZINA HUQ,¹ RUHUL A. KHAN,¹ BERNARD RIEDL,²
and MONIQUE LACROIX¹

¹Research Laboratories in Sciences Applied to Food, Canadian Irradiation Center (CIC), INRS-Institute Armand-Frappier, University of Quebec, 531 Boulevard des Prairies, Laval, Quebec H7V 1B7, Canada

²Centre de recherche sur le bois, Faculté de foresterie, de géomatique et de géographie, Université Laval, Quebec, G1V 0A6, Canada

Global environmental concern, regarding the use of petroleum-based packaging materials, is encouraging researchers and industries in the search for packaging materials from natural biopolymers. Bioactive packaging is gaining more and more interest not only due to its environment friendly nature but also due to its potential to improve food quality and safety during packaging. Some of the shortcomings of biopolymers, such as weak mechanical and barrier properties can be significantly enhanced by the use of nanomaterials such as nanocellulose (NC). The use of NC can extend the food shelf life and can also improve the food quality as they can serve as carriers of some active substances, such as antioxidants and antimicrobials. The NC fiber-based composites have great potential in the preparation of cheap, lightweight, and very strong nanocomposites for food packaging. This review highlights the potential use and application of NC fiber-based nanocomposites and also the incorporation of bioactive agents in food packaging.

Keywords Packaging materials, nanocellulose, biodegradable films, nanocomposites, bioactive polymers, biopolymers, essential oils

INTRODUCTION

The purpose of food packaging is to preserve the quality and safety of the food it contains, from the time of manufacture to the time it is used by the consumer. An equally important function of packaging is to protect the product from physical, chemical, or biological damages. The outer covering should also inform the consumer about the product. The packaging also has a secondary function, i.e., reduction of loss, damage, and waste for distributor and customer, and facilitates its storage, handling, and other commercial operations. About 50% of agricultural products are destroyed because of the absence of packaging. The causes of this loss are bad weather and physical, chemical, and microbiological deteriorations. Progress in the packaging of foodstuffs will prove crucial over the next few years mainly because of new consumer patterns, demands creation, and world population growth which is estimated to be 15 billion by 2025. The most well-known packaging materials that meet

these criteria are polyethylene- or copolymer-based materials, which have been in use by the food industry for over 50 years. These materials are not only safe, inexpensive, versatile, but also flexible. However, one of the limitations with plastic food packaging materials is that it is meant to be discarded, with very little being recycled (Cha and Chinnan, 2004; Villanueva et al., 2006). Currently, almost all the plastics, which are widely used in the various sectors, are produced from petrochemical products. With rising petroleum costs, there is concern with finding cost-effective ways to manufacture packaging materials. In addition to the above environmental issues, food packaging has been impacted by notable changes in food distribution, including globalization of the food supply, consumer trends for more fresh and convenient foods as well as a desire for safer and better quality foods. Given these and previously mentioned issues, consumers are demanding that food packaging materials be more natural, disposable, potentially biodegradable as well as recyclable (Chandra and Rustgi, 1998; Fischer et al., 1999).

Bio-based packaging is defined as packaging containing raw materials originating from agricultural sources, i.e., produced from renewable, biological raw materials such as starch, cellulose, and bio-derived monomers. To date, biodegradable packaging has commanded great attention, and numerous projects

Address correspondence to Monique Lacroix, Research Laboratories in Sciences Applied to Food, Canadian Irradiation Center (CIC), INRS-Institute Armand-Frappier, University of Quebec, 531 Boulevard des Prairies, Laval, Quebec H7V 1B7, Canada. E-mail: monique.lacroix@iaf.inrs.ca

are under way in this field. One important reason for this attention is the marketing of environmentally friendly packaging materials. Furthermore, use of biodegradable packaging materials has the greatest potential in countries where landfill is the main waste management tool. Bio-based packaging materials include both edible films and edible coatings along with primary and secondary packaging materials (Siro and Plackett, 2010; Khan et al., 2010b). Unfortunately, so far the use of biodegradable films for food packaging has been strongly limited because of the poor barrier properties and weak mechanical properties shown by natural polymers. For this reason natural polymers were frequently blended with other synthetic polymers or, less frequently, chemically modified with the aim of extending their applications in more special or severe circumstances (Weber et al., 2002; Khan et al., 2010a).

Cellulose is one of the most abundant biopolymers on earth, occurring in wood, cotton, hemp, and other plant-based materials and serving as the dominant reinforcing phase in plant structures. Plant fibers are mainly composed of cellulose, hemicellulose, and lignin. Cellulose, which awards the mechanical properties of the complete natural fiber, is ordered in microfibrils enclosed by the other two main components: hemicellulose and lignin (Bledzki & Gassan 1999). Cellulose microfibrils can be found as intertwined microfibrils in the cell wall (2–20 nm diameter and 100–40,000 nm length depending on its source). In these microfibrils, there exist nanofibers (also composed by cellulose) with diameters of 5–50 nm and lengths of several millimetres conformed by nanocrystalline domains and amorphous regions (Darder et al., 2007). Cellulose is a linear carbohydrate polymer chain consisting of D-glucopyranose units joined together by β -1,4-glycosidic linkages. In the unit cell of cellulose, two chains are joined by hydrogen bonding to each other in a parallel conformation, which is called cellulose. These units are packed side-by-side to form microfibrils of cellulose, which also contain disordered or amorphous regions. The arrangement of the cellulose microfibrils in the primary wall is random. Secondary cell walls of plants contain cellulose (40–80%), hemicellulose (10–40%), and lignin (5–25%), where cellulose microfibrils are embedded in lignin. Hemicellulose is a highly branched polymer compared to the linearity of cellulose. Its structure contains a variety of sugar units, whereas cellulose contains only 1,4- β -D-glucopyranose units and its degree of polymerization is 10–100 times lower than that of cellulose. Finally, lignin is a complex hydrocarbon polymer with both aliphatic and aromatic constituents (Soykeabkaew et al., 2008).

The cellulose molecules are always biosynthesized in the form of nanosized fibrils, which are in turn assembled into fibers, films, walls, etc. The cellulose nanofibers are called nanocellulose (NC). The molecular arrangements of these fibrillar bundles are so small that the average diameter of the bundle is about 10 nm. These cellulose nanofibers are with diameters of 5–50 nm and lengths of thousands of nanometers. NC is a cellulose derivative composed of a nanosized fiber network which determines the product properties and its functionality. NC fibers are very interesting nanomaterials for production of

cheap, lightweight, and very strong nanocomposites. Generally, NC is produced by the bio-formation of cellulose via bacteria and also by the disintegration of plant celluloses using shear forces in refiner techniques. Wood-derived NC can also be prepared by electrospinning from pulp solutions (Dufresne, 1997) or by controlled acid hydrolysis of wood pulp (Beck-Candanedo et al., 2005). Cellulose nanofibers are recognized as being more effective than their microsized counterparts to reinforce polymers due to interactions between the nanosized elements that form a percolated network connected by hydrogen bonds, provided there is a good dispersion of the nanofibers in the matrix. It is predicted that NC reinforcements in the polymer matrix may provide value-added materials with superior performance and extensive applications for the next generation of biodegradable materials. NC is expected to show high stiffness since the Young's modulus (YM) of the cellulose crystal is as high as 134 GPa. The tensile strength of the crystal structure was assessed to be approximately 0.8 up to 10 GPa (Cao et al., 2008; Dieter-Klemm et al., 2009; Azeredo et al., 2010).

Polymer composites are mixtures of polymers with inorganic or organic additives having certain geometries (fibres, flakes, spheres, particulates). The use of nanoscale fillers is leading to the development of polymer nanocomposites and represents a radical alternative to the conventional polymer composites ((Dieter-Klemm et al., 2006). Polymer nanocomposites have generated enormous interest since Toyota[®] researchers in the late 1980s showed that as little as 5% addition of nanosized clays to nylons greatly increased their modulus and heat distortion temperature (Kojima et al., 1993). The use of nanocomposites serve a number of important functions, such as extending the food shelf life, enhancing food quality because they can serve not only as barriers to moisture, water vapor, gases, and solutes, but also serve as carriers of some active substances, such as antioxidants and antimicrobials (Rhim and Hong, 2006). These nanocomposites are significant due to their nanoscale dispersion with size less than 1,000 nm (Sanguansri and Augustin, 2006). Addition of relatively low levels of nanoparticles (less than 5%) have been shown to substantially improve the properties of the finished plastic, increasing the deformability and strength, and reducing the electrical conductivity and gas permeability (Sorrentino and Gorrasi, 2007).

The review discusses potential use, application, and advantages of nanocomposites, especially nanocellulose in the field of food packaging. This review highlights the potential of biopolymers (alginate, chitosan, etc.) for food packaging and also the incorporation of bioactive agents or antimicrobials (organic acids, bacteriocins, essential oils, etc.) into packaging to improve the quality and safety of food products during storage.

NANOCOMPOSITES

Nanocomposites are mixture of polymers with nanosized inorganic or organic fillers with particular size, geometry, and surface chemistry properties. The polymers used are normally

hydrocolloids, such as proteins, starches, pectins, and other polysaccharides. Various inorganic nanoparticles have been recognized as possible additives to enhance the polymer performance (John and Thomas, 2008). Nanofillers include solid layered clays, synthetic polymer nanofibers, cellulose nanofibers, and carbon nanotubes. Up to now, only the layered inorganic solids like layered silicate have attracted the attention of the packaging industry. This is due to their ready availability and low cost, and also their significant enhancement of finished product properties and relative simple processing (Sorrentino and Gorrasi, 2007).

Advantages of Nanocomposites

When polymers are combined with nanofillers, the resulting nanocomposites exhibit significant improvements in mechanical properties, dimensional stability, and solvent or gas resistance with respect to the pristine polymer. Owing to the nanosize particles obtained by dispersion, these nanocomposites can exhibit many advantages such as biodegradability, enhanced organoleptic characteristics of food, such as appearance, odor, and flavor; reduced packaging volume, weight, and waste; extended shelf life and improved quality of usually nonpackaged items; individual packaging of small particulate foods, such as nuts and raisins; function as carriers for antimicrobial and antioxidant agents; controlled release of active ingredients; annually renewable resources (Hitzky et al., 2005; Rhim, 2007).

Nanocomposites also offer extra benefits like low density, transparency, good flow, better surface properties, and recyclability. The enhancement of many properties resides in the fundamental length scales dominating the morphology and properties of these materials. The nanofiller particles have at least one dimension in the nanometer (from 1 to 100 nm) range. It means that a uniform dispersion of these particles can lead to ultra-large interfacial area between the constituents. The very large organic or inorganic interface alters the molecular mobility and the relaxation behavior, improves the mechanical properties of nanocomposites both in solid and melt states, and the thermal stability and melt viscosity of renewable polymers also increase after nanocomposite preparation (Han and Floros, 1997; Penner and Lagaly, 2001; Sorrentino and Gorrasi, 2007). Manias et al. (2001), reported that small additions—typically less than 6 wt% of nanoscale inorganic fillers could promote concurrently several of the polypropylene material properties, including improved tensile characteristics, higher heat deflection temperature, retained optical clarity, high barrier properties, better scratch resistance, and increased flame retardancy. Strawhecker and Manias (2000) suggested that for a 5% montmorillonite (MMT) exfoliated composite, the softening temperature of nanocomposites increased by 25°C, the water permeability reduced by 60%, and the nanocomposites could retain their optical clarity. For these reasons, these are far lighter in weight than conventional biodegradable composites and make them competitive with other materials for specific applications,

especially food packaging (Petersen et al., 1999). Another advantage of nanocomposite is that it can be biodegraded efficiently. Degradation of a polymer may result from the action of microbes, macro-organisms, photo degradation or chemical degradation (Avella et al., 2005).

Application of Nanocomposites in Food Packaging

The use of proper packaging materials and methods to minimize food losses and provide safe and wholesome food products have always been the focus of food packaging. In addition, consumer trends for better quality, fresh-like, and convenient food products have intensified during the last decades. Therefore, a variety of active packaging technologies have been developed to provide better quality, wholesome, and safe foods, and also to limit package-related environmental pollution and disposal problems. The application of nanocomposites may open a new possibility to solve these problems. Nanocomposite packaging materials have great potential for enhanced food quality, safety, and stability as an innovative packaging and processing technology. The unique advantage of the natural biopolymer packaging may lead to new product development in food industry, such as individual packaging of particulate foods, carriers for functionally active substances, and nutritional supplements (Ozdemir and Floros, 2004).

NANOCELLULOSE (NC)

Nanocelluloses (NCs) are described as celluloses composed of nanosized fibers and nanofiber structuring which determines the product's properties. The similar term *nanosized cellulose* is used in case of isolated crystallites and whiskers formed by acid-catalyzed degradation of celluloses. This field and the application of that nanosized cellulose, e.g., in composites, have been intensively investigated. Typical examples have been reported (Ljungberg et al., 2005; Masa et al., 2005).

Types of Nanocellulose

As described above, one type of NC is formed directly as the result of biosynthesis of special bacteria, and these types of NCs are called bacterial NC. A very pure product with subsequently reported important properties is formed that necessitates challenging biosynthesis/biotechnological handling and the development of large-scale production. Another kind of NC can be prepared from the nearly inexhaustible source of feedstock wood using controlled mechanical disintegration steps to produce the favored product properties (Masa et al., 2005).

Nanocellulose from Bacteria

In 1886, A. J. Brown discovered bacterial cellulose (BC) as a biosynthetic product of *Gluconacetobacter xylinus* strains

(Klemm et al., 1998). He identified a gelatinous mass, formed on the solution during the vinegar fermentation as cellulose. It is mentioned that in the middle of the 20th century, a special culture medium was developed for *Gluconacetobacter xylinus* to optimize cellulose formation on the laboratory scale. As a result of systematic and comprehensive research over the last decade, broad knowledge of the formation and structure of BC has been acquired. This work is an important part of the integration of biotechnological methods into polysaccharide chemistry and the development of cellulose products with new properties and application potential (Tischer et al., 2011).

Nanocellulose from Wood

In contrast to BC, cellulose from wood is composed of fibers that are about 100 times thicker. Because of the complex and expensive cultivation of BC (sophisticated medium and long cultivation time), it is also a challenge to produce nanofibrillated celluloses from wood. The substructures of wood are only accessible by chemical treatment (Klemm et al., 1998) and mechanical disintegration procedures. In the last 25 years, there have been efforts to reduce wood fibers in size. As a first step, in the early 1980s, Turbak et al. (1983) developed micro fibrillated cellulose (MFC). Today, there are different ways to produce materials with controlled fiber diameters. At first, a water suspension of pulp has to go through a mechanical treatment that consists of a spring-loaded valve assembly (refiner), where the slurry is pumped at high pressure. The formed MFC is moderately degraded and extremely expanded in surface area. In recent years, cellulose with a nanoscale web-like structure has been made. The fiber diameters are in the range 10–100 nm (Nakagaito & Yano, 2004, 2005). The degree of fibrillation depends on the number of passes through the refiner. Another technique to prepare wood MFC/NC is described by Takahashi et al. (2005). The aim was the creation of strong composites in tension using hot-pressed fibers without synthetic polymers but with the original wood components hemicelluloses and lignin as binders. The starting material was bamboo because of its high cellulose content. Bamboo-fiber bundles and monofilaments were ground under high-speed conditions using stone disks. A combination of thermal and alkali pretreatments, given the appropriate ratio of cellulose, hemicelluloses and lignin in the monofilaments led to strong adhesion between the fibers under the hot-press conditions. Suzuki and Hattori (2004) treated a pulp with a solid concentration of 1–6% with a disk refiner more than 10 times. The fibers obtained had a length of less than 0.2 mm. There have also been some investigations into the properties of NC from wood, which has an amazing water-storage capacity, similar to BC. A dispersion of these cellulose fibers in water with a solid content of only 2% leads to a mechanically stable transparent gel. The wood NC fibers are suitable for solidification of emulsion paints and filter aids, useful for both primary rough filtration and precision filtration. Furthermore, NC from wood is used in paper-making as a coating

and dye carrier in paper tinting. Moreover, it can be utilized in the food industry as a thickening agent, a gas-barrier, and in moisture resistant paper laminate for packaging. In cosmetics, wood NC is suitable as an additive in skin-cleansing cloths, and as part of disposal diapers, sanitary napkins, and incontinence pads. Possible medical applications are directed to excipients such as binders, fillers, and/or disintegrants in the development of solid dosage forms (Fukuda et al., 2001; Kumar, 2002; Kyomori et al. 2005).

Besides application in its pure form, it is possible to use NC from wood in polymer composites. In embedding tests, the tensile strength of such composites was five times higher than that of the original polymers. This result, as well as its natural origin, makes this NC attractive for combination with different biopolymers. Possible applications for such reinforced biopolymers could arise in areas such as medicine, food industry, and gardening (Nakagaito and Yano, 2004, 2005). In these sectors, properties such as biodegradability, high mechanical strength, and where required optical transparency are important. It should also be mentioned that the application of wood NC prepared by the described techniques, where the cell wall is further disintegrated by mechanical treatment, leads to lower-strength cellulose fiber-reinforced composites than in the corresponding BC materials (Gindl and Keckes, 2004).

Application of Nanocellulose

In recent years due to the exceptional properties of these innovative NC polymers, many widespread utilization have been observed. Membranes and composites from cellulose and cellulose esters are important domains in the development and application of these polymeric materials. The most important segment by volume in the chemical processing of cellulose contains regenerated cellulose fibers, films, and membranes. In the case of the cellulose esters, mainly cellulose nitrate and cellulose acetate as well as novel high-performance materials are created, which are widely used as laminates, composites, optical/photographic films, and membranes, or as other separation media. The direct formation of stable and manageable BC fleeces as the result of bacterial biosynthesis in the common static culture is significant. This and their exciting properties have led to the increasing use of BC as a membrane material and composite component. Contaminations incorporated from the culture medium and bacterial cells can be removed from the BC by smooth purification methods depending on the application area. One recent example of the formation and application of foils/membranes of unmodified bacterial NC is described by George and coworkers (George et al., 2005). The processed membrane seems to be of great relevance as a packaging material in the food industry, where continuous moisture removal and minimal-oxygen-transmission properties play a vital role. The purity, controllable water capacity, good mechanical stability, and gas-barrier properties of bacterial NC are important parameters for this application.

Nanocellulose Based Composites

There have been several researches on the use of NC as a reinforcing agent in polymer matrices. Nanocomposites based on nanocellulosic materials have been prepared with petroleum-derived nonbiodegradable polymers such as polyethylene (PE) or polypropylene (PP) and also with biodegradable polymers such as polylactic acid (PLA), polyvinyl alcohol (PVOH), starch, polycaprolactone (PCL), methylcellulose, and chitosan.

Bruce et al. (2005) prepared composites based on Swede root MFC and different resins including four types of acrylic and two types of epoxy resins. All the composites were significantly stiffer and stronger than the unmodified resins. The main merit of the study was that it demonstrated the potential for fabricating nanocomposites with good mechanical properties from vegetable pulp in combination with a range of resins. Apart from good mechanical properties, high composite transparency can be important for some applications (e.g., in the optoelectronics industry). Iwamoto et al. (2005, 2008) reported that because of the size of nanofibers reinforced acrylic resin retains the transparency of the matrix resin even at fiber contents as high as 70 wt%. The BC with nanofiber widths of 10 nm also has potential as a reinforcing material for transparent composites. As for example, Nogi et al. (2005, 2006a, 2006b) obtained transparent composites by reinforcing various acrylic resins with BC at loadings up to 70 wt% by reducing the average fiber size (diameter 15 nm). Abe et al. (2007) fabricated an NC (from wood) containing acrylic resin nanocomposite with transmittance higher than that of BC nanocomposite in the visible wavelength range and at the same thickness and filler content. This finding indicated that nanofibers obtained from wood were more uniform and thinner than BC nanofibers.

Another remarkable and potentially useful feature of NC is their low thermal expansion coefficient (CTE), which can be as low as 0.1 ppm K⁻¹ and comparable with that of quartz glass (Nishino et al., 2004). This low CTE combined with high strength and modulus could make NC a potential reinforcing material for fabricating flexible displays, solar cells, electronic paper, panel sensors and actuators, etc. As an example, Nogi and Yano (2008) prepared a foldable and ductile transparent nanocomposite film by combining low-YM transparent acrylic resin with 5 wt% of low CTE and high-YM of BC. The same researchers reported that transparent NC sheets prepared from NC and coated with acrylic resin have low CTEs of 8.5–14.9 ppm K⁻¹ and a modulus of 7.2–13 GPa (Nogi and Yano, 2009). Polyurethane (PU), which is a polar polymer, has the potential to interact with the polar groups of cellulose molecules leading to enhanced mechanical and interfacial properties of the composites. PU-MFC composite materials were prepared recently using a film stacking method in which the PU films and nonwoven cellulose fibril mats were stacked and compression moulded (Seydibeyoglu and Oksman, 2008). The thermal stability and mechanical properties of the pure PU were improved by MFC reinforcement. Nanocomposites with 16.5 wt% fibril

content had tensile strength and YM values nearly 5 and 30 times higher, respectively, than that of the corresponding values for the matrix polymer.

Polyvinyl alcohol (PVOH) is a water-soluble alcohol, which is biocompatible, biodegradable, and also has excellent chemical resistance. Therefore, PVOH has a wide range of practical applications. In particular, PVOH is an ideal candidate for biomedical applications including tissue reconstruction and replacement, cell entrapment and drug delivery, soft contact lens materials, and wound covering bandages for burn victims (Ding et al., 2004). Sun-Young Lee et al. (2009) reported the fabrication of PVOH-NC composites by the reinforcement of NC into a PVOH matrix at different filler loading levels and subsequent film casting. The NC was prepared by acid hydrolysis of MCC at different hydrobromic acid (HBr) concentration. Chemical characterization of NC was performed for the analysis of crystallinity (Xc), degree of polymerization (DP), and molecular weight (Mw). The acid hydrolysis decreased steadily the DP and Mw of MCC. The crystallinity of MCC with 1.5 M and 2.5 M HBr showed a significant increase due to the degradation of amorphous domains in cellulose. The mechanical and thermal properties of the NC reinforced PVOH films were also measured for tensile strength and thermo-gravimetric analysis (TGA). The tensile strength (TS) of pure PVOH film was 49 MPa. The TS of NC-reinforced PVOH films after 1.5 M HBr hydrolysis showed the highest value (73 MPa) at the loading of 1 wt%. This value was 49% higher than pure PVOH film. However, the NC loading of 3 and 5 wt% to PVOH matrix gradually decreased the values of TS. The TS of PVOH films with 3 and 5 wt% NC were 3.0 and 55.3% lower, respectively, compared to those with 1 wt% NC. The TGA of NC-reinforced PVOH films revealed three main weight loss regions. The first region at a temperature of 80–140°C is due to the evaporation of physically weak and chemically strong bound water, and the weight loss of the film in those ranges is about 10 wt%. The second transition region at around 230–370°C is due to the structural degradation of PVOH composite films and the total weight loss in those ranges was about 70%. The third stage weight loss occurred above 370°C, due to the cleavage backbone of PVOH composite films or the decomposition of carbonaceous matter. Wan et al. (2006) tested BC as a potential reinforcing material in PVOH for medical device applications. These authors developed a PVOH-BC nanocomposite with mechanical properties tuneable over a broad range, thus making it appropriate for replacing different tissues. A number of applications using MFC for reinforcing PVOH have been reported. For example, Zimmermann et al. (2004) dispersed MFC into PVOH and generated fibril-reinforced PVOH nanocomposites (fibril content 20 wt%) with up to three times higher YM and up to five times higher TS when compared to the reference polymer. A blend containing 10% NC obtained from various sources, such as flax bast fibers, hemp fibers, kraft pulp or rutabaga and 90% PVOH was used for making nanofiber-reinforced composite material by a solution casting procedure (Bhatnagar and Sain, 2005). Both TS and YM were improved compared to neat PVOH film, with a pronounced four- to five-fold increase in YM observed. Poly(caprolactone)

(PCL), a biodegradable polymer, is suitable as a polymer matrix in biocomposites. Lönnberg et al. (2008) prepared MFC-grafted PCL composites via ring-opening polymerization (ROP). This changes the surface characteristics of MFC, for grafting made it possible to obtain a stable dispersion of MFC in a nonpolar solvent. It also improved the compatibility of MFCs with PCL. The thermal behavior of MFC grafted with different amount of PCL has been investigated using thermal gravimetric analysis (TGA) and differential scanning calorimetry (DSC). The crystallization and melting behavior of free PCL and MFC-PCL composites were studied with DSC, and a significant difference was observed regarding melting points, crystallization temperature (T_g), degree of crystallinity, as well as the time required for crystallization.

Khan et al. (2010c) prepared methylcellulose (MC)-based films casted from its 1% aqueous solution containing 0.5% vegetable oil, 0.25% glycerol, and 0.025% Tween80[®]. Puncture strength (PS), puncture deformation (PD), viscoelasticity coefficient, and water vapor permeability (WVP) were found to be 147 N/mm, 3.46 mm, 41%, and 6.34 g.mm/m².day.kPa, respectively. Aqueous NC solution (0.1–1%) was incorporated into the MC-based formulation, and it was found that both PS and WVP values were improved by 117 and 26%, respectively. Films containing 0.25% NC were found to be the optimum. Khan et al. (2010c) also reported the effect of gamma radiation on the NC containing MC-based composites. The films were irradiated from 0.5 to 50 kGy doses, and it was revealed that mechanical properties of the films were slightly increased at low doses because of NC fibers reorientation, whereas barrier properties were further improved to 29% at 50 kGy.

Dufresne and Vignon (1998, 2000) prepared potato starch-based nanocomposites, while preserving the biodegradability of the material through addition of MFC. The cellulose filler and glycerol plasticizer content were varied between 0–50 wt% and 0–30 wt%, respectively. MFC significantly reinforced the starch matrix, regardless of the plasticizer content, and the increase in YM as a function of filler content was almost linear. The YM was found to be about 7 GPa at 50 wt% MFC content compared to about 2 GPa for unreinforced samples (0% MFC). However, it was noted that when the samples were conditioned at high relative humidity (75% RH), the reinforcing effect of the cellulose filler was strongly diminished. Since starch is more hydrophilic than cellulose, in moist conditions it absorbs most of the water and is then plasticized. The cellulosic network is surrounded by a soft phase and the interactions between the filler and the matrix are strongly reduced. Besides improving mechanical properties of starch, addition of MFC to the matrix resulted in a decrease of both water uptake at equilibrium and the water diffusion coefficient. Nanocomposites from wheat straw nanofibers and thermoplastic starch from modified potato starch were prepared by the solution casting method (Alemdar and Sain, 2008). Thermal and mechanical performance of the composites was compared with the pure thermoplastic starch (TPS) using TGA, dynamic mechanical analysis (DMA), and

tensile testing. The TS and YM were significantly enhanced in the nanocomposite films, which could be explained by the uniform dispersion of nanofibers in the polymer matrix. The YM of the TPS increased from 111 to 271 MPa with maximum (10 wt%) nanofiber filling. In addition, the glass transition (T_g) of the nanocomposites was shifted to higher temperatures with respect to the pure TPS. Azeredo et al. (2010) developed NC-reinforced chitosan films with different NC and glycerol (plasticizer) content. They evaluated the effect of different concentration of NC and glycerol on the TS, YM, T_g, elongation at break (Eb), and WVP of the chitosan-based composite films. They have an optimum condition of 18% glycerol and 15% NC, based on the maximization of TS, YM, T_g, and decreasing WVP values while maintaining a acceptable Eb of 10%. Pereda et al. (2010) developed sodium caseinate films with NC by dispersing the fibrils into film forming solutions, casting, and drying. Composite films have been reported to be less transparent and had a more hydrophilic surface than neat sodium caseinate films. However, the global moisture uptake was almost not affected by the NC concentration. Addition of NC to the neat sodium caseinate films produced an initial increase in the WVP and then decreased as filler content increased. The TS and TM of the composite films have been reported to increase significantly with a more than two times increase in TS and TM than the native films at 3% NC content.

CLASSIFICATION OF BIOPOLYMERS

A vast number of biopolymers or biodegradable polymers are chemically synthesized or biosynthesized during the growth cycles of all organisms. Some micro-organisms and enzymes capable of degrading them have been identified (Averous & Boquillon, 2004). Figure 1, proposes a classification with four different categories, depending on the synthesis:

- (a) Polymers from biomass such as the agro-polymers from agro-resources, e.g., starch, cellulose.
- (b) Polymers obtained by microbial production, e.g., poly(hydroxyalkanoates).
- (c) Polymers chemically synthesized using monomers obtained from agro-resources, e.g., poly(lactic acid).
- (d) Polymers whose monomers and polymers are both obtained by chemical synthesis from fossil resources, e.g., poly(caprolactone), polyester amide, etc.

Except the fourth family, which is of fossil origin, most polymers of family (a)–(c) are obtained from renewable resources (biomass). The first family is agro-polymers (e.g., polysaccharides) obtained from biomass by fractionation. The second and third families are polyesters, obtained respectively by fermentation from biomass or from genetically modified plants (e.g., polyhydroxyalkanoate) and by synthesis from monomers obtained from biomass (e.g. polylactic acid). The fourth family

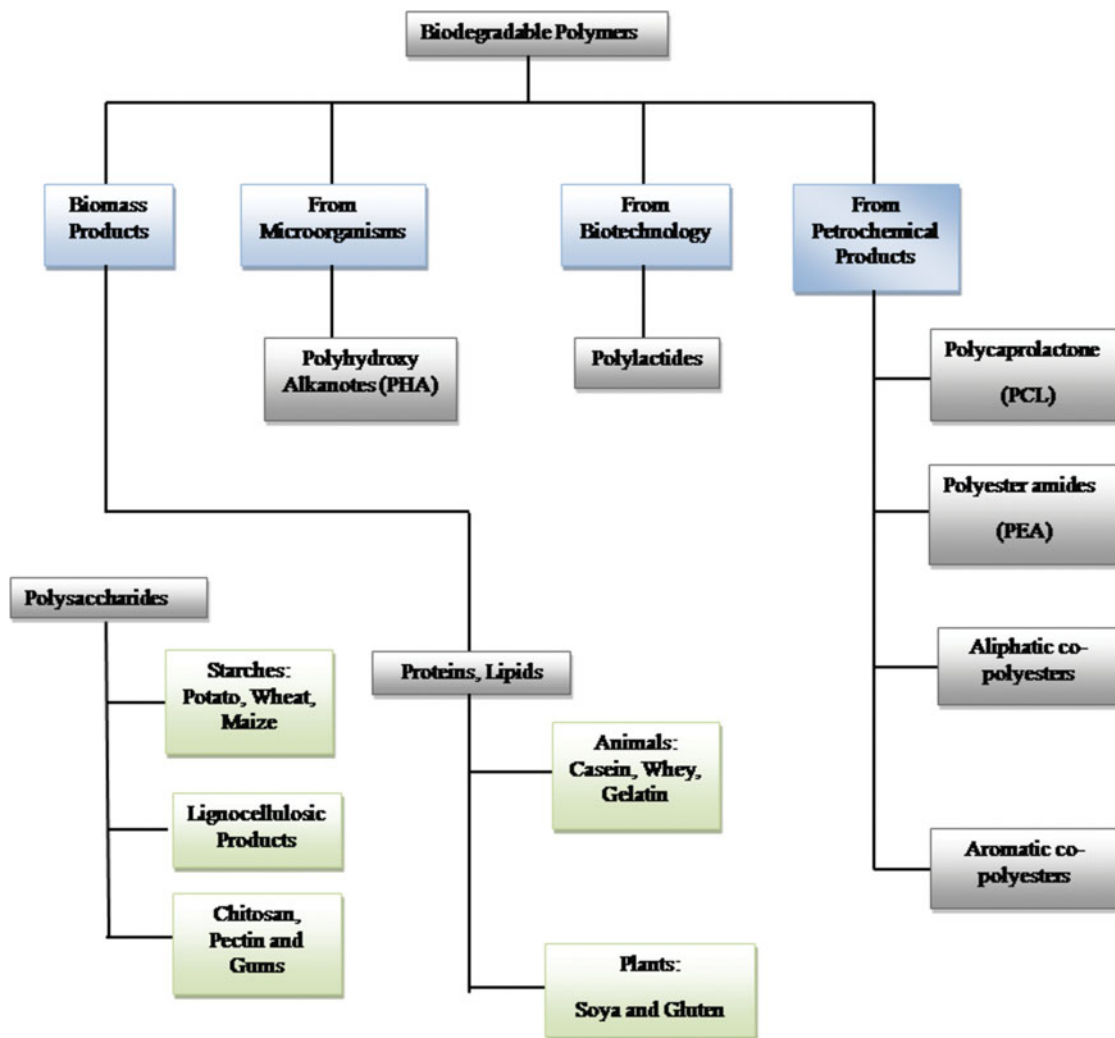


Figure 1 Classification of biodegradable polymer (adopted from John & Thomas, 2008). (Color figure available online.)

is polyesters, totally synthesized by the petrochemical process (e.g., polycaprolactone; polyester amide; aliphatic or aromatic copolyesters). A large number of these biopolymers are commercially available. They show a large range of properties and they can compete with nonbiodegradable polymers in different industrial fields (John and Thomas, 2008).

BIOACTIVE PACKAGING

Bioactive packaging is gaining interest from researchers and industries due to its potential to provide quality and safety benefits. The reason for incorporating bioactive agents into the packaging is to prevent surface growth of micro-organisms in foods where a large portion of spoilage and contamination occurs (Appendini and Hotchkiss, 2002; Coma, 2008). This approach can reduce the addition of larger quantities of antimicrobials that are usually incorporated into the bulk of the food. A controlled

release from packaging film to the food surface has numerous advantages over dipping and spraying. In the latter processes, in fact, antimicrobial activity may be rapidly lost due to inactivation of the antimicrobials by food components or dilution below active concentration due to migration into the bulk food matrix (Janjarasskul and Krochta, 2010). Numerous researchers have demonstrated that bioactive polymers such as, alginate, chitosan, gelatine, etc., and antimicrobial compounds such as organic acids (acetic, propionic, benzoic, sorbic, lactic, lauric), potassium sorbate, bacteriocins (nisin, lacticin), grape seed extracts, spice extracts (thymol, p-cymene, cinnamaldehyde), thio-sulfonates (allicin), enzymes (peroxidase, lysozyme), proteins (conalbumin), isothiocyanates (allylisothiocyanate), antibiotics (imazalil), fungicides (benomyl), chelating agents (ethylenediaminetetraacetic acid-EDTA), metals (silver), or parabens (heptylparaben) could be added to edible films to reduce bacteria in solution, on culture media, or on a variety of muscle foods (Cutter, 2002 and 2006). A short discussion on some of the bioactive polymers and bioactive agents is given here:

Bioactive Polymers

Bioactive polymers such as, alginate, chitosan, gelatin, etc., can be used for the packaging of food products. Alginates are linear copolymers of β -(1-4)-linked D-mannuronic acid and α -(1-4)-linked L-guluronic acid units, which exist widely in many species of brown seaweeds. Since it was discovered by Stanford in 1881, alginate has been used in a wide range of industries, such as food, textile printing, paper and pharmaceuticals, and for many other novel end-uses (Khan et al., 2010b). Study found that alginate coatings retarded oxidative off-flavors, improved flavor, and juiciness in re-heated pork patties (Earle & McKee, 1976). Other researchers have extended the shelf life of shrimp, fish, and sausage with alginate coatings (Cutter and Samner, 2002). Sodium alginate coatings extended the shelf life of salted and dried mackerel (Jo et al., 2001). Chitosan is a linear polysaccharide consisting of 1, 4-linked 2- amino-deoxy- β -D-glucan, is a deacetylated derivative of chitin, which is the second most abundant polysaccharide, found in nature after cellulose. Chitosan has been found to be nontoxic, biodegradable, biofunctional, biocompatible, and was reported by several researchers to have strong antimicrobial and antifungal activities. Chitosan has been compared with other biomolecule-based active films used as packaging materials and the reported results showed that chitosan has more advantages because of its antibacterial activity and bivalent minerals chelating ability (Chen et al., 2002). Chitosan films have been successfully used as a packaging material for the quality preservation of a variety of foods (Ouattara et al., 2000). Antimicrobial films have been prepared by including various organic acids and essential oils in a chitosan matrix, and the ability of these bio-based films to inhibit the growth of indigenous (*Lactic acid bacteria* and *Enterobacteriaceae*) or inoculated bacteria (*Lactobacillus sakei* and *Serratia liquefaciens*) onto the surfaces of vacuum-packed cured meat products have been investigated. Release of organic acids (acetic and propionic) was found to be initially fast, when the gradient of ion concentration between the inside of the polymer matrix and the outside environment was high, then decreased as the release of acids progressed. At the same time, it was shown that the antimicrobial activity of the bio-based films under study did not affect growth and activity of lactic acid bacteria, whereas the growth of *Enterobacteriaceae* and *S. liquefaciens* was delayed or completely inhibited after storage during 21 days at 4°C (Quintavalla and Vicini, 2002). Recently, a chitosan–starch film has been prepared using microwave treatment which may find potential application in the food packaging technology (Dutta et al., 2009; Aider, 2010). Chitosan films have been made via treatments with various acids and incorporated into packaging films for processed meats and seafood, as well as combined with nisin and coated onto the surfaces of paper for inhibiting microorganisms (Vartiainen et al., 2004). Durango et al. (2006) also developed and evaluated an edible film made from 3% or 5% chitosan and starch against *S. enteritidis* in suspensions. When applied directly to cell suspensions, 1% chitosan reduced the pathogen $>4\log_{10}$ CFU/mL (or

99.99%). Subsequent experiments demonstrated that chitosan-treated films made with 3% or 5% chitosan reduced populations of *S. enteritidis* $> 1\log_{10}$ CFU/mL (or 90%). The authors demonstrated that chitosan-treated films made with 5% chitosan were the most efficient treatment for inhibiting *S. enteritidis* in solution and that the application of these films to foodstuffs was in progress. In another study, Cooksey (2005) incorporated nisin into chitosan to inhibit *L. monocytogenes*. In solution and in agar diffusion assays, the antimicrobial film inhibited the pathogen, but no further studies were conducted in meat systems (Cha and Chinnan, 2004).

Organic Acids

Organic acids, such as acetic, benzoic, lactic, citric, nalidixic, maleic, tartaric, propionic, fumaric, sorbic, etc., are one of the most common ingredients used for bioactive packaging. Yamanaka et al. (2000), described the influence of bioactive organic agents such as nalidixic acid as additives to the bacterial cellulose (BC) culture medium. In that case, not only the crystallization of the fibers and the material properties were influenced but the *Gluconacetobacter* cells were also changed. Using antibiotics in a concentration of 0.1 mM, a 2–5 times elongation of the cell length was observed due to inhibition of cell division. The fibers became 1–2 times wider compared to common BC. Ghosh et al. (1977), developed fungistatic wrappers with sorbic acid and applied them to bread. This wrapper necessitated heating the wrapped bread at 95–100°C for a period of 30 to 60 minutes. The incorporation of an antioxidant in the treated wrapper and also the use of an odor adsorbent inside the bread packs minimized off-flavor development. Sliced bread, based on sensory evaluation, was found acceptable up to 1 month, and as a sandwich up to 3 months. The fungistatic wrappers were made by coating grease-proof paper with an aqueous dispersion of sorbic acid in 2% carboxymethyl cellulose solution. Using this sorbic acid-treated paper and then enclosing the food in a polyethylene bag could preserve foods that are generally amenable to spoilage by mold for minimum of 10 days. Han and Flores (1997) studied the incorporation of 1.0% w/w potassium sorbate in low density polyethylene films. A 0.1-mm-thick film was used for physical measurements. It was found that potassium sorbate lowered the growth rate and maximum growth of yeast, and lengthened the lag period before mold growth became apparent. Weng et al. (1999) developed the technique of combining polyethylene-co-methacrylic acid (PEMA) with benzoic and sorbic acid to form antimicrobial food packaging material. Devlieghere et al. (2000) studied the antimicrobial activity of ethylene vinyl alcohol (EVA)/linear low density polyethylene (LLDPE) containing potassium sorbate. Because of the limited migration of K-sorbate from LLDPE film, the inhibition effect of this film against *Candida* spp., *Pichia* spp., *Trichosporon* spp., and *Penicillium* spp. appeared very weak. Moreover, no significant differences could be observed for yeast and mold growth on the cheese cubes

compared to a reference film during storage of cheese packaged in a *K*-sorbate film. Benzoic anhydride-incorporated antimicrobial polyethylene films and minimal microwave heating were used to control the microbial growth of Tilapia fish fillets.

Bacteriocins

The bacteriocin such as nisin, which is produced by the lactic acid bacterium, *Lactococcus lactis*, is one of the most effective agents when it comes to antimicrobial packaging. It is the most effective against lactic acid bacteria and other gram-positive organisms, notably the *Clostridia* species (Jin and Zhang, 2008). Imran et al. (2010) developed hydroxypropyl methylcellulose films with nisin and evaluated the antimicrobial activity of the films against *Listeria*, *Staphylococcus*, *Enterococcus*, and *Bacillus* strains. It has been reported that film bioactivity demonstrated efficacy against *Listeria* > *Enterococcus* > *Staphylococcus* > *Bacillus* spp. Scannell et al. (2000) developed bioactive food packaging materials using immobilized nisin and lactacin 3147. The antimicrobial packaging reduced the lactic acid bacteria counts in sliced cheese and ham at refrigeration temperatures, thus, extending the shelf life. Nisin adsorbed bioactive inserts reduced levels of *Listeria innocua* by below 2 log units in cheese and ham and *Staphylococcus aureus* in cheese (~1.5 log units) and ham (~2.8 log units). Ming et al. (1997) applied nisin and pediocin to cellulose casings to reduce *L. monocytogenes* in meats and poultry. Pediocin-coated bags completely inhibited the growth of inoculated *L. monocytogenes* through 12 weeks storage at 4°C. Pediocin is another bacteriocin, which was found to be effective against *L. monocytogenes*. Wilhoit (1996 and 1997) has received a patent for the method of employing pediocin-coated cellulose casings on meat for inhibiting the growth of *L. monocytogenes*. Cutter and Siragusa (1997) reported that immobilization of the bacteriocin nisin in calcium alginate gels not only resulted in greater reductions of bacterial populations on lean and adipose beef surfaces, but also resulted in greater and sustained bacteriocin activity when the tissues were ground and stored under refrigerated conditions for up to 7 days, as compared to nisin-only controls.

Essential Oils and Plant Extracts

The antimicrobial activity of essential oils and plant extracts has been recognized for many years. Ouattara et al. (2001) evaluated the combined effect of low-dose gamma irradiation and protein-based coatings with thyme oil and trans-cinnamaldehyde to extend the shelf life of pre-cooked shrimp. The product's shelf life was significantly extended without altering the appearance and taste of shrimp for thymol treatment concentrations of up to 0.9%. Oussalah et al. (2007) developed alginate-based edible films with 1% (w/v) essential oils of Spanish oregano (O; *Corydothymus capitatus*), Chinese cinnamon (C; *Cinnamomum cassia*), or winter savory (S; *Satureja mon-*

tana) to control pathogen growth on bologna and ham slices. The bologna and ham slices were inoculated with *Salmonella* Typhimurium or *Listeria monocytogenes* at 10³ CFU/cm². On bologna, C-based films were the most effective against the growth of *Salmonella* Typhimurium and *L. monocytogenes*. *L. monocytogenes* was the more sensitive bacterium to O-, C-, and S-based films. *L. monocytogenes* concentrations was found to be below the detection level (<10 CFU/mL) after five days of storage on bologna coated with O-, C-, or S-based films. On ham, a 1.85 log CFU/cm² reduction of *Salmonella* Typhimurium ($P \leq 0.05$) have been reported after five days of storage with C-based films. *L. monocytogenes* was highly resistant in ham, even in the presence of O-, C-, or S-based films. However, C-based films were the most effective against the growth of *L. monocytogenes*. Oussalah et al. (2004), also developed milk protein-based edible films containing 1.0% (w/v) oregano, 1.0% (w/v) pimento, or 1.0% oregano-pimento (1:1) essential oils mix were applied on beef muscle slices. The application of bioactive films on meat surfaces containing 10³ CFU/cm² of *Escherichia coli* O157:H7 or *Pseudomonas* spp. showed that film containing oregano was the most effective against both the bacteria, whereas film containing pimento oils was reported to have least effect against these two bacteria. A 0.95 log reduction of *Pseudomonas* spp. level, as compared to samples without film, was observed at the end of storage in the presence of films containing oregano extracts. A 1.12 log reduction of *E. coli* O157:H7 level was reported in samples coated with oregano-based films. Hammer et al. (1999) investigated 52 plant oils and extracts for activity against *Acinetobacter baumannii*, *Aeromonas veronii* biogroup *sobria*, *Candida albicans*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens* and *Staphylococcus aureus*, using an agar dilution method. Lemongrass, oregano, and bay inhibited all organisms at concentrations of $\leq 2.0\%$ (v/v). Six oils did not inhibit any organisms at the highest concentration, which was 2.0% (v/v) oil for apricot kernel, evening primrose, macadamia, pumpkin, sage and sweet almond. Variable activity was recorded for the remaining oils. Twenty of the plant oils and extracts were investigated, using a broth microdilution method, for activity against *C. albicans*, *S. aureus* and *E. coli*. The lowest minimum inhibitory concentrations were 0.03% (v/v) thyme oil against *C. albicans* and *E. coli*, and 0.008% (v/v) vetiver oil against *S. aureus*. Smith-Plamer et al. (1998) investigated antimicrobial properties of 21 plant essential oils and two essences were investigated against five important food-borne pathogens, *Campylobacter jejuni*, *Salmonella enteritidis*, *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes*. The oils of bay, cinnamon, clove, and thyme were the most inhibitory, each having a bacteriostatic concentration of 0.075% or less against all five pathogens. In general, gram-positive bacteria were more sensitive to inhibition by plant essential oils than the gram-negative bacteria. *Campylobacter jejuni* was the most resistant of the bacteria investigated to plant essential oils, with only the oils of bay and thyme having a bacteriocidal concentration of less than 1%. At 35°C, *L. monocytogenes* was extremely

sensitive to the oil of nutmeg. A concentration of less than 0.01% was bacteriostatic and 0.05% was bacteriocidal, but when the temperature was reduced to 4°C, the bacteriostatic concentration was increased to 0.5% and the bacteriocidal concentration to greater than 1%.

CONCLUSION

Among the many different materials that mankind is currently dependent on, nonbiodegradable polymers are arguably still one of the most important considering their widespread usage in food packaging industries. Currently, almost all the nonbiodegradable polymers that are widely used in various sectors are produced from petrochemical products. Due to concerns for the global environment and the increasing difficulty in managing solid wastes, biodegradable polymeric materials, bio-nanocomposites, and bioactive packaging may be among the most suitable alternatives for many applications. Addition of bioactive polymers (alginate, chitosan, etc.) or bioactive agents such as organic acids, essential oils, and plant extracts, bacteriocins can significantly enhance the quality and safety of food products during storage and can also prevent the growth of microorganisms in food. Similarly, NC-based composites, due to their excellent mechanical and barrier properties and their role as the carrier of bioactive substances, have great potential in food packaging industries. The field of food packaging represents a promising and exciting field for the use of nanotechnology. Use of nanotechnology in food packaging can not only increase the mechanical and barrier properties of the films but can also increase the safety and shelf life of the packaged food products by allowing a controlled or sustained release of antimicrobials or bioactive agents. However, there has been little study on the combination of NC with bioactive agents to have composite films that will fulfill both mechanical and antimicrobial properties required for food packaging. So, composites films with both NC and bioactive agents, represent a promising field of research and should have an enormous impact on food packaging over the coming years.

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Effect of gamma radiation on the mechanical and barrier properties of HEMA grafted chitosan-based films

Avik Khan, Tanzina Huq, Ruhul A. Khan, Dominic Dussault, Stephane Salmieri, Monique Lacroix*

Research Laboratories in Sciences Applied to Food, Canadian Irradiation Center (CIC), INRS-Institut Armand-Frappier, Institute of Nutraceuticals and Functional Foods, University of Quebec, 531 Boulevard des Prairies, Laval, Quebec, Canada H7V 1B7

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ABSTRACT

Chitosan films were prepared by dissolving 1% (w/v) chitosan powder in 2% (w/v) aqueous acetic acid solution. Chitosan films were prepared by solution casting. The values of puncture strength (PS), viscoelasticity coefficient and water vapor permeability (WVP) of the films were found to be 565 N/mm, 35%, and 3.30 g mm/m² day kPa, respectively. Chitosan solution was exposed to gamma irradiation (0.1–5 kGy) and it was revealed that PS values were reduced significantly ($p \leq 0.05$) after 1 kGy dose and it was not possible to form films after 5 kGy. Monomer, 2-hydroxyethyl methacrylate (HEMA) solution (0.1–1%, w/v) was incorporated into the chitosan solution and the formulation was exposed to gamma irradiation (0.3 kGy). A 0.1% (w/v) HEMA concentration at 0.3 kGy dose was found optimal-based on PS values for chitosan grafting. Then radiation dose (0.1–5 kGy) was optimized for HEMA grafting. The highest PS values (672 N/mm) were found at 0.7 kGy. The WVP of the grafted films improved significantly ($p \leq 0.05$) with the rise of radiation dose.

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1. Introduction

Bio-based packaging is defined as packaging containing raw materials originating from agricultural sources, such as chitosan, alginate, starch, cellulose, and bio-derived monomers. Bio-based packaging materials include both edible films and edible coatings along with primary and secondary packaging materials (Siro and Plackett, 2010; Salmieri and Lacroix, 2006). Chitosan is prepared from chitin, which is the second most abundant polysaccharide, found in nature after cellulose. Chitosan is a linear polysaccharide and is composed of glucosamine and *N*-acetyl glucosamine residues with a β -1, 4-linkage. Chitosan is non-toxic, biodegradable, bio-functional, biocompatible, and have strong antimicrobial and antifungal activities (Dutta et al., 2009; Aider, 2010). Chitosan-based films were compared with other biopolymer-based active films used as packaging materials and was reported that chitosan has advantages over other bio-polymers (Kim et al., 2011).

Chitosan films have been successfully used as a packaging material for the preservation of a variety of foods quality (Chen et al., 2009). However, natural polymers such as chitosan are usually hydrophilic in nature. So, modification of chitosan is required to improve its properties. Modifications on the chitosan

structure can be carried out in order to adequate it to various applications, such as, drug carrier by *N*-acylation (Le Tien et al., 2003); for biomedical applications by graft copolymerization (Prashanth and Tharanathan, 2007), etc. The general effect of radiation on chitosan has been evaluated by Chmielewski (2010). Among the various methods of modification used to improve chitosan properties, graft copolymerization is widely used (Sun et al., 2003). The modification of polymeric materials by graft copolymerization is reported elsewhere (Khan et al., 2010; Sashiwa and Aiba, 2004) because it can provide materials with desired properties through the appropriate choice of the side chain to be grafted (Casimiro et al., 2005). Chitosan bears, two types of reactive groups, that can be modified by grafting: the C-2 free amino groups on deacetylated units and the hydroxyl groups in the C-3 and C-6 either in acetylated or deacetylated units (Berger et al., 2004). The monomer, 2-hydroxyethyl methacrylate (HEMA) is a synthetic and water soluble vinyl monomer. Singh and Ray (1994) were the first to prepare HEMA grafted chitosan films. It is reported (Sultana et al., 2010) that HEMA can cross-link with gelatin by gamma radiation and the grafted films possessed high mechanical strength. The advantage of using gamma radiation is that it does not require the addition of chemical initiators or lethal agents to promote the polymerization reaction and, at the same time, promotes the inactivation of pathogenic micro-organisms (Casimiro and Gil, 2010). The main objective of this study was to find out the suitability of gamma radiation for the preparation of HEMA grafted chitosan films for food packaging applications.

* Corresponding author. Tel.: +1 450 687 5010; fax: +1 450 686 5501.
E-mail address: monique.lacroix@iaf.inrs.ca (M. Lacroix).

2. Materials and methods

2.1. Materials

Chitosan (molecular wt. 700 kDa; degree of deacetylation 88–89%) was obtained from Kitomer Marinard (Quebec, Canada). Monomer, 2-hydroxyethyl methacrylate (HEMA) was purchased from Sigma-Aldrich Canada Ltd.

2.2. Film preparation

1% (w/v) chitosan was dissolved in 2% aqueous acetic acid solution. The chitosan solution was then γ -irradiated from 0.1 to 5 kGy. Then the irradiated solution was casted onto Petri dishes and was allowed to dry at room temperature (RH was 40–50%). The HEMA grafted chitosan films were prepared by slowly incorporating HEMA (0.1–1%, w/v) into the chitosan solution under constant stirring. The solution was stirred for 1 h and then subjected to gamma irradiation at different doses under air. After irradiation treatment, the solution was again stirred for 1 h and the films were prepared by casting.

2.3. Irradiation

Irradiation of both chitosan and chitosan/HEMA solution was conducted with γ -rays generated from a ^{60}Co source at room temperature, at a dose rate of 17.878 kGy/h in an Underwater Calibrator-15A Research Irradiator (Nordion Inc., Kanata, ON, Canada). The solutions were irradiated from 0.1 kGy to 25 kGy.

2.4. Film thickness

Film thickness was measured using a Mitutoyo Digimatic Indicator (Type ID-110E; Mitutoyo Manufacturing Co. Ltd., Tokyo, Japan) with a resolution of 0.001 mm, at five random positions around the film, by slowly reducing the micrometer gap until the first indication of contact.

2.5. Puncture strength (PS)

PS was measured by the Stevens-LFRA texture analyzer (model TA-1000; Texture Technologies Corp., Scarsdale, NY). Films were fixed between two perforated Plexiglas plates (3.2 cm diameter), and the holder was held tightly with two screws. A cylindrical probe (2 mm diameter; scale, 0–900 g; sensitivity, 2 V) was moved perpendicularly to the film surface at a constant speed (1 mm/s) until it passed through the film. Strength values at the puncture point are used to calculate the hardness of the film. The PS values were divided by the thickness of the films to avoid any variation related to this parameter. PS is calculated using the equation $\text{PS (N/mm)} = (9.81 F)/x$, where F is the recorded force value, x is the film thickness, and 9.81 is the gravitational acceleration.

2.6. Viscoelasticity coefficient (Y)

Viscoelastic properties were evaluated using relaxation curves. The same puncture test procedure described above was used, but the probe is stopped to 3 mm after film contact and maintained for 1 min. The relaxation coefficient Y is calculated using the equation:

$$Y(\%) = [(F_i - F_f) / F_i] \times 100$$

where, F_i is the initial recorded value (g) and F_f the second value measured after 1 min of relaxation. A low relaxation coefficient ($Y \rightarrow 0\%$) indicates high film elasticity, whereas a high coefficient

($Y \rightarrow 100\%$) indicates high film plasticity related to a more rigid and easily distorted material.

2.7. Water vapor permeability (WVP)

WVP tests were conducted gravimetrically using an ASTM 15.09: E96 procedure (ASTM, 1983). Films were mechanically sealed onto Vapometer cells (No. 68-1, Twining-Albert Instrument Co., West Berlin, NJ) containing 30 g of anhydrous calcium chloride (0% RH). The cells were initially weighed and placed in a Shellab 9010L controlled humidity chamber (Sheldon Manufacturing Inc., Cornelius, OR) maintained at 25 °C and 64% RH for 24 h. The amount of water vapor transferred through the film and absorbed by the desiccant is determined from the weight gain of the cell. The assemblies were weighed initially and after 24 h for all samples and up to a maximum of 10% gain. WVP is calculated according to the combined Fick and Henry laws for gas diffusion through coatings and films.

2.8. Statistical analysis

To validate the results obtained during different experimental procedure each analysis was carried out in triplicate. An analysis of variance (ANOVA) and multiple comparison tests of Duncan's were used to compare all the results. Differences between means were considered significant when the confidence interval is smaller than 5% ($p \leq 0.05$). The results were analyzed by the PASW Statistics 18 software (IBM Corporation, Somers, NY, USA).

3. Results and discussion

3.1. Effect of gamma irradiation and HEMA treatment on puncture strength

The puncture strength (PS) of control chitosan films was found to be 565 N/mm. Fig. 1 depicts the effect of gamma radiation on the PS of chitosan and HEMA grafted chitosan films. It was found that PS of irradiated chitosan films increased at low radiation doses (≤ 0.3 kGy). The PS values of the irradiated (0.3 kGy) chitosan films reached to 597 N/mm, which is 5.7% higher than control samples. The increase of PS of the films at low radiation doses may be due to the formation of dimmer (formation of chitosan oligomers) with acetic acid (Park et al., 2002). At doses > 0.3 kGy, a decrease in PS was observed. At 5 kGy, the PS of the films decreased by 47% as compared to the control, which may be due to the radiation degradation of chitosan. It was not possible to prepare chitosan films from solutions

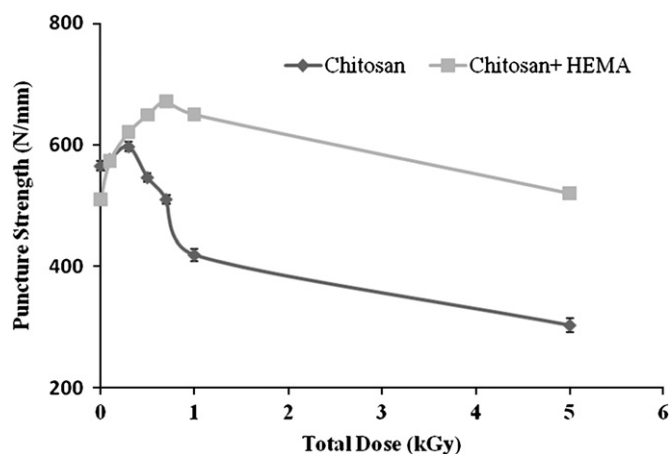


Fig. 1. Effect of gamma radiation on the puncture strength of films.

irradiated at doses higher than 5 kGy. HEMA solution (0.1–1% w/v) was incorporated into the chitosan solution then exposed to gamma radiation. It was found that films containing 0.1% (w/v) HEMA exhibited the highest PS (621 N/mm) at doses 0.3 kGy. So, the optimized HEMA concentration (0.1%, w/v) was exposed to gamma radiation at doses from 0.1 to 5 kGy. The PS of the films increased with the increase of radiation dose up to 0.7 kGy. At 0.7 kGy, HEMA grafted chitosan films exhibited a PS of 672 N/mm, which is 20% higher than that of the control chitosan films. The increase in PS may be attributed to the reaction of acrylic groups of HEMA with amino group of chitosan. However, the PS of the films sharply decreased at doses >0.7 kGy. The decrease of the mechanical strength could be due to the formation of poly(HEMA) by homo polymerization and also due to the degradation of chitosan. The glycosidic linkage of the natural polymer may generally break under gamma irradiation. At higher doses, the polymer may undergo scission and may be broken into smaller fragments. As a result, the mechanical strength of the polymer decreases (Vanichvattanadecha et al., 2010; Huang et al., 2007).

3.2. Effect of gamma irradiation on the viscoelasticity (Y) coefficient

Fig. 2 shows the effect of gamma irradiation on the viscoelasticity coefficient (Y) of the chitosan and HEMA grafted chitosan films. It was found that Y coefficient values of the chitosan films decreased significantly ($p \leq 0.05$) at doses 0.3–5 kGy. However, the Y coefficient values of the HEMA grafted chitosan films increased significantly ($p \leq 0.05$) with the increase of radiation dose up to 0.5 kGy. The Y coefficient values of the HEMA treated but non-irradiated (0 kGy) chitosan films were found to be 43%. When treated at 0.5 kGy, Y coefficient value was found to be 63% ($p \leq 0.05$). At 0.5 kGy, the Y coefficient reached to a plateau. So, it was evident that HEMA grafted chitosan films showed better elastic property than the control chitosan films.

3.3. Effect of gamma radiation on the WVP

Fig. 3 represented the effects gamma radiation on the WVP of control and HEMA treated chitosan films. The WVP of the chitosan films was found to be 3.30 g mm/m² day kPa. At 0.1 kGy, the WVP of the chitosan films decreased sharply and showed a value of 3.07 g mm/m² day kPa, which is almost 7% lower than that of the chitosan films. However, at radiation doses (> 0.1 kGy), the WVP of the films increased and at 5 kGy the WVP value was 3.41 g mm/m² day kPa. So, a very low radiation dose (0.1 kGy) contributed the chitosan films more water vapor resistant. The WVP of the HEMA treated chitosan films decreased with the increase of radiation doses.

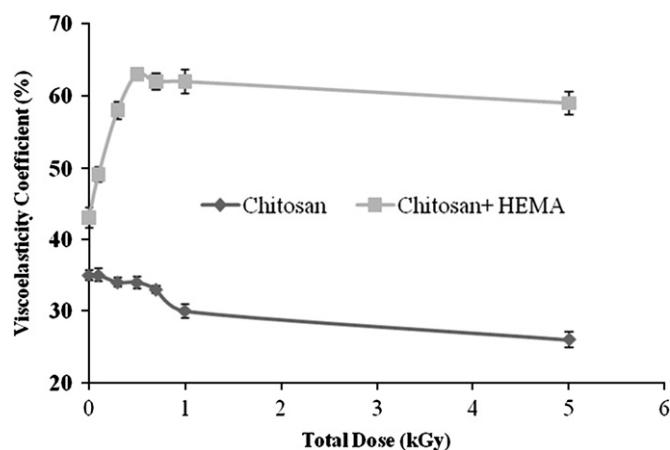


Fig. 2. Effect of gamma radiation on the viscoelasticity coefficient (%) of films.

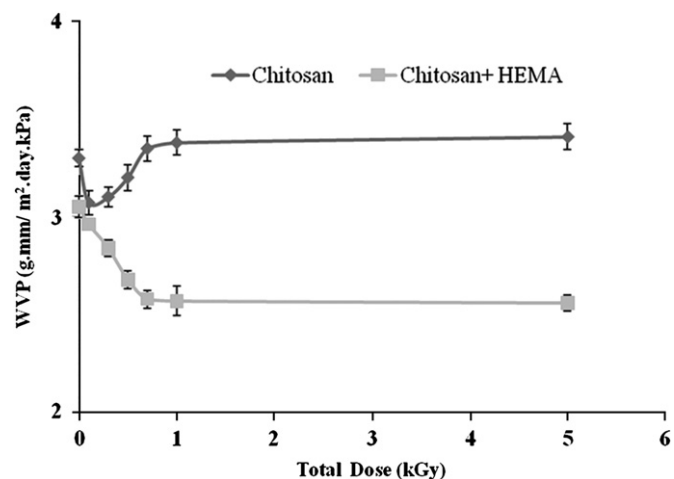


Fig. 3. Effect of gamma radiation on the WVP of films.

At 0.7 kGy the WVP of the HEMA treated chitosan films was found to be 2.58 g mm/m² day kPa. Therefore, a 22% reduction of WVP was obtained by treating chitosan with HEMA followed by the exposure of 0.7 kGy dose.

4. Conclusion

From this study it was found that low radiation doses (0.1–0.3 kGy) on chitosan solution improved the mechanical strength of the films. The HEMA grafted chitosan films possessed better mechanical and barrier properties compared to the control chitosan samples. So, gamma radiation can be considered as a safe and good source for the preparation of monomer grafted films.

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