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Faculty of Natural and Agricultural Sciences

Robustness of *Bifidobacterium longum* LMG 13197 encapsulated in lyophilized Vegetal BM 297 ATO-inulin lipid based synbiotic microparticles

BY

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Submitted in partial fulfilment of the requirements for the degree

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In the Faculty of Natural and Agricultural Sciences, Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, South Africa

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DECLARATION

I declare that the dissertation " Robustness of *Bifidobacterium longum* LMG 13197 encapsulated in lyophilized Vegetal BM 297 ATO-inulin lipid based synbiotic microparticles" which I hereby submit for the degree of Magister Scientiae at the University of Pretoria is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

....

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PUBLICATIONS AND CONFERENCE CONTRIBUTIONS

Research articles

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LIST OF ABBREVIATIONS

AMC: Arroyo, Martin and Cotton

ATR: Acid Tolerance Response

CFDA: Carboxyfluorescein diacetate

CIE: Commission Internationale de I' Eclairage

CLSM: Confocal Laser Scanning Microscopy

DCM: Dichloromethane

DNA: Deoxyribonucleic acid

DP: Degree of Polymerization

EE: Encapsulation Efficiency

EFSA: European Food Safety Authority

ENM: Electronic Nose Methodology

FAO: Food and Agricultural Organisation

FCA: Flow Cytometry Assay

FDA: Food and Drug Administration

FISH: Fluorescent In-Situ Hybridization

FMA: Fluorochrome Microplate Assay

FOS: Fructooligosaccharides

FSC: Forward Angle Light Scatter

GBH: Glyceryl Behenate

GC: Guanine and Cytosine

GIT: Gastrointestinal Tract

GMP: Genetically Modified Probiotics

GMS: Glyceryl Mono-Stearate

GOS: Galactooligosaccharides

GRAS: Generally Regarded as Safe

HLB: Hydrophillic-Lipophillic Balance

HPLC: High Performance Liquid Chromatography

ICH: International Conference on Harmonization

LAB: Lactic Acid Bacteria

LDL: Low-Density Lipoprotein

LSD: Least Significant Difference

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MRS: De Man, Rogosa and Sharpe Agar

NADH: Nicotinamide Adenine Dinucleotide

NDO: Non Digestible Oligosaccharide

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PI: Propidium Iodide

PVA: Polyvinyl Alcohol

PVAc-CA: Poly- (vinyl acetate co-crotonic acid)

PVP: Poly (vinyl pyrrolidone)

ScCO_2: Supercritical Carbon dioxide

SEM: Scanning Electron Microscopy

SGF: Simulated Gastric Fluid

SIF: Simulated Intestinal Fluid

SSC: Side Angle Light Scatter

USDA: United States Department of Agriculture

VPC: Viable Plate Count

WHO: World Health Organization

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SUMMARY

Robustness of *Bifidobacterium longum* LMG 13197 encapsulated in lyophilized Vegetal BM 297 ATO-inulin lipid based synbiotic microparticles

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Consumers are currently concerned about improving their health, and therefore demand foods that are beneficial to overall health. This has caused the rising interest in probiotics, which are live microorganisms which when ingested in sufficient amounts, restore balance in the gastrointestinal tract and consequently improve health. Probiotic bacteria have been incorporated into various food products which are now referred to as functional foods, and represent about 65% of the world's food market. Probiotics are sensitive to various environmental factors such as oxygen, moisture, pH and temperature. It is of great importance that probiotics remain viable and alive throughout the stages of processing, storage in food products and during gastrointestinal transit in order for them to confer health benefits. The use of prebiotics and microencapsulation to protect and ensure viability of probiotics has been used in food industries. Challenges faced when using most microencapsulation techniques include the need for a food grade encapsulating material, stability of the probiotic cells during encapsulation processes and storage, the need to minimize negative effects they might have on the organoleptic properties of foods into which they are incorporated. The freeze drying technique, which is known to be suitable for the preservation of probiotic cells, avoids heat induced injuries to cells and also slows down detrimental chemical reactions, was used in the current study to prepare microparticles encapsulating probiotic bifidobacteria. Due to limited reports on the use of lipid based food

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grade encapsulating materials for the microencapsulation of probiotics, this study explored the use of such materials and developed a lipid based synbiotic material which is expected to protect and improve probiotic viability.

A lipid based excipient Vegetal BM 297 ATO and various concentrations of the prebiotic inulin were used to prepare different formulations, followed by an investigation to determine which concentration of inulin resulted in better protection and survival of *Bifidobacterium longum* LMG 13197 during the freeze drying process. *Bifidobacterium longum* LMG 13197 was successfully encapsulated in Vegetal using freeze drying method. It was observed that the formulation prepared with 2% (w/v) inulin resulted in better protection of *B. longum* LMG 13197 during the encapsulation process. Characterization of the microparticles revealed that they contained high numbers of bacterial cells resulting from relatively high encapsulation efficiency. The presence of inulin resulted in microparticles with an acceptable size which is desirable for food applications. These results led to further investigation of the potential of Vegetal-inulin matrix to protect bifidobacteria in simulated gastrointestinal fluids and improve shelf life under different storage conditions.

This study demonstrates that the Vegetal-inulin matrix protected *B. longum* LMG 13197 during transit in the simulated gastric fluid (SGF) and subsequently released the cells in the simulated intestinal fluid (SIF). In comparison with the unencapsulated cells, the number of cells released in SIF was higher, which suggests that the Vegetal-inulin matrix has the potential to release probiotics in the colon for health benefits to be exerted. The shelf life of encapsulated *B. longum* LMG 13197 powders stored in glass bottles was investigated under two different storage temperatures for 6 weeks. The study demonstrates that although there was a high loss of viable probiotic cells during storage at 25°C, Vegetal-inulin matrix improved survival of probiotics for 3 weeks as opposed to the unencapsulated cells. On the other hand, encapsulation with Vegetal did not offer improved survival of bacteria when compared to the unencapsulated cells at 4°C, but the addition of inulin offered better protection for up to 5 weeks. Therefore, better shelf life of Vegetal-inulin microparticles containing *B. longum* LMG 13197 can be achieved at 4°C than at 25°C.

The food industry experience challenges in maintaining probiotic viability in dairy products due to post acidification processes occurring at refrigeration temperatures. The potential of Vegetal-inulin matrix to protect bifidobacteria in yoghurt during storage was investigated. It

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is also important that the microparticles containing probiotics do not negatively affect the organoleptic properties of the food product into which they are incorporated. In light of this, this study also investigated how the incorporation of Vegetal-inulin microparticles containing bifidobacteria affects the organoleptic properties of yoghurt. The amount of viable bacterial cells obtained at the end of storage period was high for Vegetal-inulin encapsulated *B. longum* LMG 13197, as opposed to the unencapsulated cells. At the end of storage period, a significant decrease in pH was observed in yoghurt containing unencapsulated bacteria than those containing encapsulated bacteria. This suggested that encapsulation prevented the release of metabolites produced by probiotics from negatively affecting the properties of yoghurt during storage. After investigating the effects of incorporated microparticles on the colour of yoghurt, a yellowish-white colour was observed for all the yoghurt samples, regardless of the significant differences observed for the colour attributes between all yoghurt samples during storage.

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INTRODUCTION

In the early 20th century, the Russian biologist Eli Metchnikoff mentioned that maintenance of the human gastrointestinal tract (GIT) requires constant ingestion of fermented food products, which contain commensal microorganisms (Holzapfel et al., 1998; Jankovic et al., 2010). The increasing need for foods that are able to contribute to better health by consumers has led to continuous research which focuses on improving the gastrointestinal balance by using live microbes, which are termed probiotics. Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). The ingestion of probiotics is associated with health benefits such as immune modulation, alleviation of lactose intolerance, acute gastroenteritis and rotavirus diarrhoea, inflammatory diseases such as Crohn's disease and ulcerative colitis, hypercholesterolemia, antibiotic-associated diarrhoea and atopic disorders (dermatitis and allergies) (Mack et al., 1999; Vesa et al., 2000; Fooks and Gibson, 2002; Furrie et al., 2005; Shah, 2007; Henker et al., 2008; Burgain et al., 2011). Factors such as stress, antibiotics and surgery can cause an imbalance in the gut system, as they tend to increase the concentration of harmful microbes while decreasing the commensals (Cummings and Macfarlane, 1997). Therefore, in order to restore balance in the gut, probiotics have to be introduced as dietary supplements or as functional foods (foods which contain live microorganisms) such as yoghurt, cheese, ice cream, chocolate and cereals (Levy, 2000; Anal and Singh, 2007). For them to promote health benefits in individuals, probiotics must be viable and able to reproduce in the human GIT, must not be toxic, must compete and exert an antagonistic effect on pathogens and must be genetically stable (Huis in't Veld et al., 1994; Lee and Salminen, 1995). The most commonly used probiotic bacteria belong to the genera *Lactobacillus* and *Bifidobacterium* (Tannock, 2001). These bacteria are preferred because they are of human origin, thus they are the only strains that can colonize and adhere to the GIT for an extended period of time (Bielecka et al., 2002).

Studies have shown that probiotics can exert health benefits if they survive the adverse conditions witnessed during manufacturing processes, storage temperatures and transit through the GIT (Brinques et al., 2011). In essence, probiotic foods should contain an adequate number of viable cells at the time of consumption, with a minimum of 10^6 – 10^7 viable cells per gram or millilitre (Ishibashi and Shimamura, 1993; Krasaekoopt et al., 2003).

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Global regulatory requirements for probiotic have become stricter over the years; as most probiotic products do not meet the standard required for beneficial purposes, due to poor survival of probiotics in food products (Doleyres and Lacroix, 2005; De Vos et al., 2010). The European Food Safety Authority (EFSA) has also stated the possibility of improper referencing and characterisation of strain for which the claims are made. Therefore, in order for manufacturers to state health claim for their probiotic products, they must have scientific proof and take into account the function and viability of the probiotic as a food additive (Jankovic et al., 2010; Burgain et al., 2011).

Among various methods applied to improve growth and survival of probiotics, the use of prebiotics has been explored and results have demonstrated improved probiotic viability in food products as well as during gastrointestinal transit (Fooks and Gibson, 2002). Prebiotics are non-digestible foods which positively affects the host by specifically stimulating the growth and activity of beneficial bacteria in the colon while restricting the harmful ones (Sekhon and Jairath, 2010). Studies have shown that inulin type prebiotics (e.g. fructooligosaccharides and inulin) have been well explored because of their ability to resist hydrolysis by gastric and pancreatic enzymes (Ramchandran and Shah, 2010). It has also been reported that addition of prebiotics improved viability of probiotic cells without affecting the sensorial properties of food (Hansen et al., 2002; Dianawati et al., 2013; Shamekhi et al., 2013).

In addition to the use of prebiotics, an approach geared towards providing a physical barrier to shield the cells from adverse environmental conditions such as light, oxygen and moisture is of considerable interest (Burgain et al., 2011). Microencapsulation is such an approach currently used in food and pharmaceutical industries to protect probiotics (Adhikari, 2000; Picot and Lacroix, 2004; Thantsha et al., 2009). It is a mechanical process which involves packaging solid, liquid and gaseous substances into small capsules (Champagne and Fustier, 2007). Therefore, microencapsulation ensures stabilization of the core material, controls oxidative reactions, masks flavours, colours or odours and extends shelf life of the active compounds (Anal and Singh, 2007). The microencapsulation techniques used include among others, extrusion and emulsion, spray dying, freeze drying and preparation of microparticles under supercritical carbon dioxide conditions (Champagne and Fustier, 2007; Thantsha et al., 2009; Burgain et al., 2011). However, the use of microencapsulation poses a lot of difficulties, as some of the techniques require the use of water, toxic organic solvents and

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high temperatures, which are all detrimental to the probiotic cells, and thus complicates food production (Champagne and Fustier, 2007; Burgain et al., 2011). Furthermore, microencapsulation requires use of food grade coating materials which are FDA (Food and Drug Administration) approved and are generally regarded as safe (GRAS) (Lian et al., 2003; Picot and Lacroix, 2004). Freeze drying has been utilized as a long term preservation technique for the encapsulation of probiotics (Petrovic et al., 2007). This method involves freezing of the culture and removal of water by sublimation under high vacuum (Carvalho et al., 2004). Freeze drying has been associated with the formation of ice crystals which cause damage to the cell structure as a result of changes in fatty acid and protein structure (Chen et al., 2006). Thus, cryoprotectants such as glucose, lactose, skim milk and fructose have been employed to prevent cell damage, thereby enhance viability of probiotics during various methods of encapsulation (Capela et al., 2006). Studies have shown that freeze drying reduces oxidative reactions, since it occurs in a vacuum (Carvalho et al., 2004). Thus, successful encapsulation of probiotics using various coating materials has been achieved using freeze drying (Capela et al., 2006; Heidebach et al., 2010; Pop et al., 2012; Chen et al., 2013). However, the use of lipid materials for the encapsulation of probiotics for food applications has not been much explored. In the current study, Vegetal BM 297 ATO will be used as a potential lipid material for the encapsulation of *Bifidobacterium longum* LMG 13197.

Following the successful encapsulation of *B. longum* LMG 13197 within Vegetal-inulin matrix by freeze drying, the specific aims of this study are to characterize the microparticles obtained after microencapsulation for their use in food industries, to assess the survival of bacteria within Vegetal-inulin microparticles when exposed to simulated gastrointestinal fluids, different storage temperatures, in yoghurt and the effects of microparticle incorporation on the physico-chemical properties of yoghurt. This study will expand the knowledge on the use of lipid matrices in food industries. It will furthermore create new ways of successfully storing probiotic supplements, should the probiotics show improved shelf life at non refrigeration temperatures. Following the survival of encapsulated probiotics and improved organoleptic quality in yoghurt, it is expected that this study will enhance the levels of live probiotic organisms in yoghurt, as well as increase the acceptability of yoghurt by consumers.

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CHAPTER 1

Literature Review

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1.1 Overview

Over the years, research and food industries have contributed to the maintenance of a healthy life style by increasing the awareness about functional foods. These functional foods contain commensal microorganisms which are associated with various health benefits (Hylemon and Harder, 1998; Hooper et al., 2002; Tuohy et al., 2003; Backhed et al., 2005; Prakash et al., 2011). The beneficial roles of these commensal microorganisms can be maintained in the gastrointestinal tract (GIT) by constant ingestion of good bacteria, which are now known as probiotics (Parvez et al., 2006).

1.2 Probiotics

1.2.1 Definitions and characteristics

Recently, a proper definition of probiotics describes them as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2002). This definition suggests that the potential probiotic microorganism must have the following characteristics; be viable after industrial processing and after long periods of storage, be certified generally regarded as safe (GRAS), originate from the gut (improves their functionality) (Hoolihan, 2001); be able to fight off pathogens; remain viable and functional after passage through the GIT and provide beneficial effects to the individual. The first actual introduction of probiotics was done by Metchnikoff in the early 1900s, where he reported that Bulgarian peasants were much healthier and lived longer as a result of the large consumption of fermented milk, due to the health benefits carried out by the live microorganisms (Metchnikoff, 1907). In order to provide individuals with the required health benefits, probiotics are supplied as supplements or incorporated into a large variety of food products such as dairy products (yoghurt, cheese and ice-cream) and non-dairy products (cereals, non-fermented juice and sausages) (Cherie et al., 1998).

There have been debates as to what amount of probiotics is required for beneficial effects to occur. It has been reported that ingesting 100 g of the product containing a minimum amount of 10^6 to 10^7 viable cells/g daily will produce beneficial effects (Boylston et al., 2004). However, levels ranging from 10^6 to10^8 cfu/ml have been reported as the required dose for

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functional properties of probiotics to take effect (Sanders and Veld, 1999; Vasiljevic and Shah, 2008). It is also important to note that the efficacy of probiotics is attributed to the different strains used, since no universal strain has been reported to provide all the proposed health benefits (Song et al., 2012). Although microorganisms like *Saccharomyces cerevisiae* and *Escherichia coli* have been used as probiotics, the lactic acid bacterial strains are the most recognised because they can serve a dual function of being food fermenters as well as health providers (Song et al., 2012). Therefore, the most utilised probiotic strains include *Lactobacillus* and *Bifidobacterium* strains. These strains are currently introduced into fermented food products, and reports have shown that they greatly reduce the severity of some gastrointestinal diseases (Anal and Singh, 2007).

1.2.2 Lactobacilli

This is the largest group of probiotic bacteria inhabiting the intestine. Lactobacilli species are described as Gram positive, acid tolerant and non spore formers which prefer anaerobic conditions but at times, they can be aerotolerant (Vasiljevic and Shah, 2008). Lactobacilli species are obligate fermenters which utilise carbohydrates as a source of energy to produce various end products (Serino et al., 2009). They have a DNA composition of $< 54\%$ mol GC content. This group consists of *Lactobacillus acidophilus, L. casei, L. salivarius, L. brevis, L. plantarum*, *L. delbrueckii ssp. bulgaricus* and gram positive cocci *Streptococcus thermophilus* (Sekhon and Jairath, 2010). These strains have been successfully isolated from the human intestines and have a GRAS status (Fooks and Gibson, 2002). Lactobacilli are commonly used as starter cultures in the production of yoghurt and other fermented products, and have also been incorporated into food products for beneficial purposes (Vasiljevic and Shah, 2008). They also release various enzymes and vitamins into intestine which improves digestion and produces lactic acid (Parvez et al., 2006). The functional benefits of the above mentioned strains have been studied and reported (Vasiljevic and Shah, 2008). Functions include providing balance to the gut by competing with disease causing pathogens for villi attachment sites and nutrients, strengthening immunity and alleviating various digestive diseases symptoms for example lactose intolerance, constipation and ulcerative colitis (Guarner and Malagelada, 2003). The above mentioned functions attributed to *Lactobacillus*, also apply to *Bifidobacterium*.

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1.2.3 Bifidobacteria

Bifidobacteria are gram positive, rod shaped, anaerobic, non gas producing organisms with a bifurcated morphology, hence the initial name *Bifidobacterium bifidus*. Bifidobacteria were first isolated from the faeces of breast fed infants by Tissier in 1960 (Novik et al., 2001). They are also classified as non-spore forming, non-motile bacteria which contribute to carbohydrate fermentation in the colon with the help of an enzyme fructose-6-phosphate phosphoketolase. This fermentation enables the production of acetic and lactic acids which are effective at eliminating pathogenic organisms (Sidarenka et al., 2008). Bifidobacteria grow at optimum temperatures of 37°C to 40°C and survive at an optimum pH of 6.5 to 7.0 (Reyed, 2007). In order to achieve proper isolation and cultivation of bifidobacteria in the laboratory, a complex organic media is needed. Hence, the selective medium chosen must be able to produce high recovery of bifidobacteria from pure or mixed cultures (Payne et al., 1999). Selective media like Modified De Man Rogosa Sharpe (supplemented with cysteine-HCL) and Arroyo, Martin and Cotton (AMC) have been recommended for the enumeration of bifidobacteria from pure and mixed cultures respectively (Arroyo et al., 1994; Payne et al., 1999). Since bifidobacteria are difficult to isolate and grow in laboratories, they have been assigned different genera in the past. These genera include *Bacillus, Bacteroides, Corynebacterium, Nocardia and Lactobacillus* (Fooks and Gibson, 2002). Bifidobacteria were accepted as a separate genus in 1974 and since then, they have been characterised based on their biochemical, morphological and DNA composition (Vasiljevic and Shah, 2008). Bifidobacteria belong to the family Actinomycete as a result of their high GC content (> 50 mol %). They make up to about more than 95% of the intestinal flora of healthy and well breast fed newborns (Lourens-Hattingh and Viljoen, 2001). This percentage increases their chances of producing beneficial health effects; as a result, they have been thoroughly researched.

Various in vitro and animal tests done on bifidobacteria, as well as their supplemented food products have shown the ability of bifidobacteria to activate immune system and alleviate digestive problems like constipation and diarrhoea (Payne et al., 1999). Studies show that health was improved because the proliferation of harmful microorganisms such as *Clostridium perfringens* and *Staphylococcus aureus* was reduced (Reyed, 2007). Just like the lactobacilli, strains of bifidobacteria are specific for the health benefits they provide.

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Therefore, this has led to the classification of all the bifidobacteria strains based on the health benefits they provide. Recently, 32 species of *Bifidobacterium* were identified from human and animal hosts. Species colonizing the human host are *B. bifidum*, *B. longum*, *B. infantis B. adolescentis, B. angulatum, B. catenulatum, B. breve* and *B. pseudocatenulatum,* while those associated with the animal hosts include *B. animalis,* B. *pseudolongum* and *B. thermophilus* (Vasiljevic and Shah, 2008*;* Sidarenka et al., 2008).

Strains like *B. bifidum* and *B. longum* are of great interest because, unlike *B. lactis*, they show low tolerance to various stress factors (Ruiz et al., 2011). *Bifidobacterium longum* are gram variable cells with either long, club or dumb shaped rods which may show bifurcation. They produce convex colonies that are shiny, soft or moist (Boylston et al., 2004). *Bifidobacterium longum* ferments glucose to produce lactic and acetic acid without gas. They are anaerobes and grow at optimum temperatures between 36-38°C, but do not grow at temperatures 20°C or 47°C (Boylston et al., 2004). The genome sequence of *B. longum* has been studied and it reveals that it carries four copies of 16s rRNA gene (Schell et al., 2002). The information provided by the genome sequence can help optimise and control their growth (Song et al., 2012). *Bifidobacterium longum* has a GRAS status and their beneficial effects have been widely studied. However, most strains of *Bifidobacterium* exhibit low tolerance to stressful factors, such as those seen in the acidic environment of the GIT and in dairy products (Anal and Singh, 2007; Ruiz et al., 2011).

1.2.4 Health benefits of probiotics

Researchers have shed a lot of light on the various benefits of probiotics. Varying effects are witnessed depending on the metabolic processes of the probiotic strain used (Oelschaeger, 2010; Burgain et al., 2011). Some of the health benefits which are attributed to probiotics include relief from lactose intolerance (Buller and Grand, 1990; Shah, 1993; Vesa et al., 2000; Hoolihan, 2001; Gibson and Rastall, 2004; Suvarna and Boby, 2005), antibiotic associated diarrhoea (Shornikova et al., 1997a, 1997b; Guandalini et al., 2000; Vanderhoof, 2000; Fooks and Gibson, 2002; Reid et al., 2003; Tuohy et al., 2003; Sullivan and Nord, 2005; Sazawal et al., 2006; Vasiljevic and Shah, 2008; Prakash et al., 2011), inflammatory bowel diseases (Sartor, 1995; Gupta et al., 2000; Papadakis and Targan, 2000; Marteau, 2001; Loftus and Sandborn, 2002; Podolsky, 2002; Itzkowitz and Harpaz, 2004; Sartor,

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2004; Furrie et al., 2005; Serino et al., 2009; Burgain et al., 2011; Prakash et al., 2011) prevention from cancer (Benno and Mitsuoka,1992; Roberfroid, 2000; Saarela et al., 2000), aiding digestion by degrading complex carbohydrates, prevent antibiotic induced diarrhoea, decrease the levels of free radicals, chelate metal ions and scavenge reactive oxygen species like hydrogen peroxide (Nutraceutix, 2001) and reducing serum cholesterol levels (hypercholesterolemia) (Fooks et al., 1999; Liong and Shah, 2005b; Begley et al., 2006; Shah, 2007).

1.2.5 Mechanism of action of probiotics

The method in which probiotics confer these beneficial effects still needs to be studied. However, it is speculated that probiotics confer these benefits through three mechanisms, namely nutrient competition, release of biochemical agents and inhibition of the interaction between pathogens and the intestinal mucosa (Fooks and Gibson, 2002; Corcionivoschi et al., 2010).

Probiotic strains confer protective mechanisms by stimulating and enhancing protective functions in the intestinal epithelium (biochemical inhibition). They perform this biochemical inhibition by binding to and secreting various bactericidal substances such as lactic acid, hydrogen peroxide, bacteriocins and certain short chain fatty acids in the intestinal mucosa (Vasiljevic and Shah, 2008). These compounds exhibit antagonistic effects on pathogenic microbes and they act to reduce the pH levels in the intestine, block bacterial adhesion to epithelial cells, make nutrients unavailable, hence making it difficult for enteropathogens such as *E. coli*, *Bacillus* and *Listeria* to survive (Vasiljevic and Shah, 2008; Corcionivoschi et al., 2010). In vitro studies have revealed the ability of certain strains of *Lactobacillus* and *Bifidobacterium* to inhibit the activity of pathogenic microorganisms. Corcionivoschi et al. (2010) reported that administration of *Lactobacillus rhamnosus* HN001 in animals increased immune response against *Salmonella enterica* infection. However, human cell lines which mimic intestinal barrier are being investigated for the proper explanation of how probiotics attaches to intestinal epithelium to exert these functions (Louvard et al., 1992).

The gut flora comprises of microorganisms which constantly compete for the available nutrients. Depending on the location of microbes in the gut, nutrients may be available in

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large quantities or in limited quantities. These commensal microbes are able to adhere to intestinal walls, hence prevent the adherence of pathogenic microbes (Prakash et al., 2011). The ability of commensal strains to adhere and compete for nutrients increases the chances of exerting beneficial effects (Tuomola et al., 1999).

The inherent capacity of probiotic strains to alter the immune system is essential for prevention and alleviation of digestive disorders. The presence of immunoglobulin A and an increasing macrophagic activity helps prevent the invasion of pathogenic microbes (Burgain et al., 2011). Also, specific toll-like receptors and dendritic cells present in the gut epithelial cells recognise secreted products of probiotics which may trigger an immune cascade such as upregulating anti-inflammatory cytokine production and phagocytic activity, which in turn leads to modulation of the immune system (Fooks and Gibson, 2002; Vasiljevic and Shah, 2008). Studies have shown the ability of *B. lactis* HN019 and *L. rhamnosus* HN001 to stimulate or reduce the activity of cytotoxic lymphocyte, hence playing a significant role in the prevention of malignant tumours (Corcionivoschi et al., 2010).

Recently, the idea of creating genetically modified probiotics (GMP) for beneficial effects by manipulating the gut flora is an increasing topic of interest (Corcionivoschi et al., 2010). Molecular techniques involving genetic screening can provide information on probiotic strains with the ability to demonstrate multiple beneficial effects. The production of genetically modified probiotic is facing rejection by the public due to the unknown effects they may exert when introduced into an unfamiliar or uncontrolled environment (Verrips et al., 1996). Rijnen et al. (1999) investigated the introduction of a gene encoding glutamate dehydrogenase, which is involved in catabolism of *Peptostreptococcus asaccharolyticus* in *Lactobacillus lactis*, in order to improve the ability of this microorganism to produce alpha ketoglutarate from glutamate, an amino acid compound present in high amounts in cheese. Since different probiotic strains are known to exert different beneficial effects due to their unique properties, characteristics and spectrum of action, more research is needed to properly identify each unique probiotic strain, their mechanisms of action and target functions as accepted relevant to health.

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1.2.6 Steps taken to improve probiotic viability

Probiotic strains must be present at a minimum concentration of 10^6 cfu/ml in probiotic products and at the time of consumption for beneficial effects to be exerted in the colon (Sanders, 1999; Vasiljevic and Shah, 2008; Lee, 2009). These viable probiotics must be active and stable throughout the storage period of the product (Sanders, 1999). Studies have reported lower counts of probiotics in products even before the expiry date, hence; the cells die before they can provide health benefits to individuals (Suita-Cruce and Goulet, 2001). These low counts are as a result of the stressful factors encountered during manufacturing, storage, transport and during gastrointestinal transit (Thantsha et al., 2009; Brinques et al., 2011). Most probiotic strains, especially bifidobacteria are sensitive to certain internal and external stress factors (Lourens-Hattingh and Viljoen, 2001; Moolman et al., 2006). These factors include competing microorganisms, gastric acid and bile concentrations in the stomach and intestines, temperature, oxygen, light, moisture and certain solvents (Lian et al., 2002; Matilla-Sandholm et al., 2002; Piano et al., 2006; Thantsha, 2007). The sensitivity of probiotic strains to these factors makes it difficult for them to reach the colon in a stable and viable state. It is important to note that the survival of probiotics depends on the ability of a particular strain or species to tolerate or resist these stress factors (Lian et al., 2002).

In order to improve probiotic viability within food products, manufacturers have incorporated additional cells (up to 200%) into products at the time of production to make up for the loss of cells encountered during storage. This practice has, however led to an increase in cost of products (Porubcan et al., 1975). In addition to making up for loss in cells, the exclusion of oxygen has been explored by packaging the probiotic products in oxygen impermeable containers (Desmond et al., 2002). Dave and Shah (1997) showed that yoghurt inoculated with *Lactobacillus and Bifidobacterium* strains and stored in glass bottles had a 30-70% survival rate than those stored in plastic containers. This is because glass bottles are better equipped to exclude oxygen than plastic containers (Dave and Shah, 1997).

Another method which involves a two step fermentation process has been explored (Picot and Lacroix, 2004). During fermentation of dairy products, starter cultures tend to grow faster and produce acids which can decrease probiotic viability. Studies have shown that probiotic

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viability was increased when an initial fermentation was done by probiotics and a second fermentation was done by starter cultures (Lankaputhra and Shah, 1997).

In another procedure, strains are carefully selected and exposed to various stress factors to increase their survival and improve the chances of applying them in food production (Ruiz et al., 2011). Studies have revealed that various molecular, morphological, biochemical and physiological changes occur during stress adaptation of strains (Ahn et al., 2001). During oxygen exposure, probiotic strains undergo cell surface component modifications, as well as the detoxification of hydrogen peroxide by NADH oxidase as was observed in *B. lactis* Bb12 (Shin and Park, 1997). During heat shock conditions, studies have revealed gene alterations and induction of chaperones as a way of counteracting heat stress (Figure 1.1). These mechanisms of evading heat stress were seen in *B. longum* NCC 2705 and *B. breve* UCC2003 (Rezzonico et al., 2007). During acid stress, bacterial strains such as *B. longum* and *B. lactis* negate acid by using an Acid Tolerance Response (ATR), as well as a key enzyme F_0F_1-ATPase, which helps pump out protons from the cytoplasm (Figure 1.1) (Sánchez et al., 2007). Furthermore, during exposure to bile salts, bifidobacteria adapt by using efflux pumps to remove or detoxify bile from the cell membrane (Figure 1.1). In the case of *B. lactis*, bile adaptation was accrued to increased production of bile salt hydrolase, an enzyme which deconjugates bile salt (Grill et al., 2000; Gueimonde et al., 2009). These experiments provided strains with improved resistant phenotypes against stressful conditions and thus they can be applied to probiotic products.

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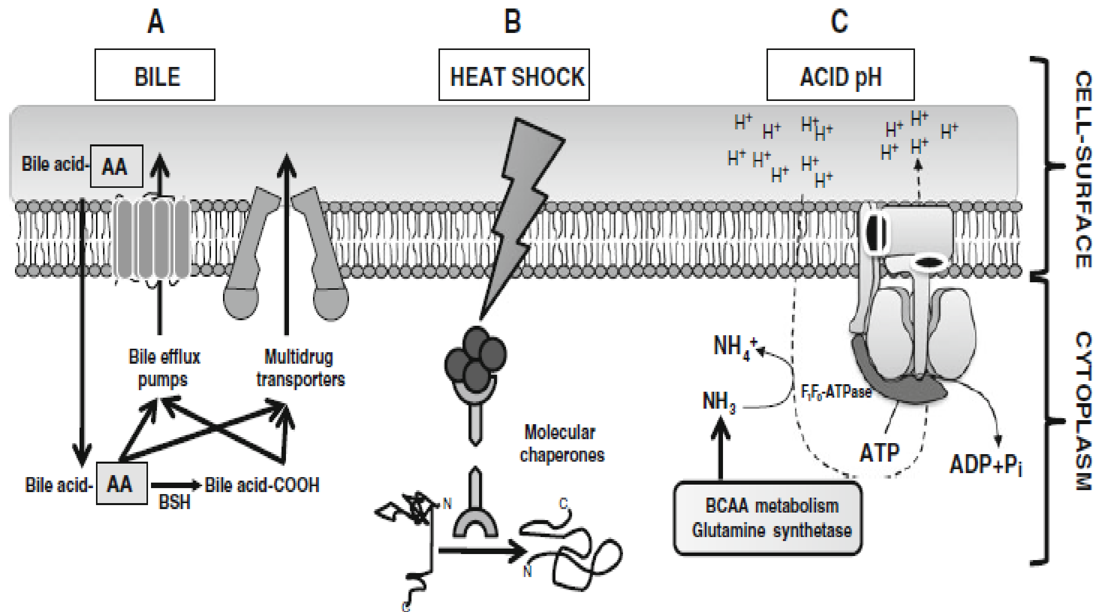

Figure 1.1: Main molecular mechanisms involved in the response of bifidobacteria to bile, heat and acidic stress conditions (Ruiz et al., 2011)

In addition to the aforementioned methods used to maintain the shelf life of probiotics, the use of prebiotics to improve probiotic viability has shown increasing interest (Gibson and Roberfroid, 1995; Sultana et al., 2000; Chávez and Ledeboer, 2007; Burgain et al., 2011; Okuro et al., 2013).

1.3 Prebiotics

Prebiotics are non-digestible foods which positively affect the host by specifically stimulating the growth and activity of beneficial bacteria in the colon while restricting the harmful ones (Fooks and Gibson, 2002; Sekhon and Jairath, 2010). In essence, the viability of probiotic is improved in products as well as in the GIT (Fooks and Gibson, 2002). It has been reported that the efficiency of prebiotics towards improving the growth of probiotics depends on the type of prebiotics and the amount used (Heydari et al., 2011). Gibson and Roberfroid (1995) reported that a substance is classified as a prebiotic if it has the following properties: it cannot be hydrolysed in the upper part of the GIT; it activates/stimulates the growth of commensal microbes and provides health benefits to the host. Since prebiotics are not living microorganisms, there is no problem with regards to their survival in the gut and in the product (Reyed, 2007). Prebiotics have been associated with improving gastrointestinal

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diseases such as constipation, irritable bowel syndrome, improving the immune system, reducing plasma triacylglycerols and absorbing divalent cations (Fiordaliso et al., 1995; Gibson and Roberfroid, 1995; Kleessen et al., 1997; Schley and Field, 2002; Cummings et al., 2003; Kelly et al., 2005; Macfarlane et al., 2007).

Some prebiotics termed non-digestible oligosaccharides/carbohydrates (NDO) are naturally occurring in food ingredients. These carbohydrates are also termed bifidogenic oligosaccharides because they selectively activate the growth of beneficial microbes (Gibson and Roberfroid, 1995). They are found in foods such as oats, legumes, artichokes, garlic, onion, banana and tomato (Macfarlane et al., 2007). Non digestible oligosaccharides are composed of 2-10 monomers which are covalently linked (Serino et al., 2009), soluble in water and in ethanol at pH 2, able to resist hydrolysis by the saliva and small intestinal enzymes, thus are accessible to the colonic bacteria for fermentation (Kelly, 2008, 2009) and their high molecular weight improves product viscosity as well their mouthfeel (Loo et al., 1999). They are low in calories, hence are used to make low caloric and diabetic food products (Serino et al., 2009). In order to increase the amount of prebiotics ingested, they are extracted from foods and used as additives in commonly purchased and ingested foodstuffs such as cereals, biscuits and other carbohydrate containing foods (Taylor et al., 1999). These oligosaccharides include among others, galacto-oligosaccharide (GOS), fructo-oligosaccharide (FOS) and inulins (Inulin type prebiotics), xylo-oligosaccharide, lactulose, isomalto-oligosaccharide and transgalacto-oligosaccharide (Holzapfel and Schillinger, 2002; Macfarlane et al., 2007).

Galacto-oligosaccharides are produced from lactose by the enzyme beta galactosidase. This enzyme produces various chain lengths with different glycosidic links such as β- (1, 4), β- (1, 3), β- (1, 2), and β- (1, 6) linkages (Gibson and Roberfroid, 1995; Cummings and Roberfroid, 1997; Fooks and Gibson, 2002). They have been incorporated into various dairy products as texture and taste enhancers due to their high solubility and good moisture retention abilities (Heavey et al., 2003; Moro et al., 2006). Malto-oligosaccharides are composed of α- D glucose molecules which are linked by α- (1-4) glycosidic linkages. They are produced by firstly treating starch molecules with isoamylase, followed by treatment with α- amylase in order to hydrolyse α- (1-4) linkages, thus producing oligosaccharides with varying lengths (Crittenden and Playne, 1996). Malto-oligosaccharides have been reported to promote the growth of bifidobacteria in human gut, while reducing the levels of *Clostridium perfringens*

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(Crittenden and Playne, 1996). Fructo-oligosaccharides comprise of one molecule of glucose and one to three molecules of fructose. The fructose molecules are linked by β-(2-1) glycosidic bonds with a terminal glucose unit (Rossi et al., 2005). These linked fructose molecules classifies FOS as an inulin prebiotics belonging to a larger group called fructans (Kelly, 2008, 2009). FOS also contain β-D-fructosyl units representing short chain (oligofructose) and medium chain (Inulin) lengths with degree of polymerization of up to 90 and 60 respectively (Bouhnik et al., 1999; Bengmark et al., 2001; Macfarlane et al., 2007). They are produced enzymatically from sucrose through transfructosylation by the help of transfructosylases and by the controlled hydrolysis of inulin from chicory (Venter, 2007). As part of the inulin type prebiotics, they play a major role in the food industry with regards to providing an increased number of probiotics for health benefits (Gibson et al., 1995).

Inulin belongs to a larger group called fructans, because they have naturally occurring oligo or polysaccharides which contains one or more β-(2-1) fructosyl-fructose linkages with mostly glycosidic bonds (Kelly, 2008). These linkages increase resistance to hydrolysis by saliva and small intestine enzymes. Hence, inulin can travel the upper GIT and reach the colon intact where it undergoes fermentation by colonic bacteria (Gibson and Roberfroid, 1995). Colonic bacteria like bifidobacteria have high enough activity of β - fructosidase which selectively breaks down glycosidic bonds, releasing fructose for their own growth (Gibson et al., 1995; Bouhnik et al., 1999). Inulin is classified into subgroups based on their degree of polymerisation (number of repeat units in an oligomer or polymer chain) (Kelly, 2008). Inulin prebiotics are produced from plant sources containing fructans, such as chicory roots and sucrose. The application of various processing technologies can produce a number of different inulin type fructans with varying strengths, weaknesses and degrees of polymerization (DP), which include oligofructose and Inulin HP (Kelly, 2008, 2009). An example is the production of high molecular weight inulin from chicory roots by hot water extraction, which produces DP ranging from 2-60 (Kelly, 2008, 2009; Kleesen et al., 1997). Fructans with DP greater than 10 are termed long chain inulin type fructans, while fructans with DP less than 10 are termed short chain inulin type fructans. Inulins can be used as ingredients in functional foods as well as dietary supplements, because they are regarded safe for consumption and they also improve texture of food (Kelly, 2008, 2009). They are found naturally in onions, garlic, wheat, banana, leeks and artichokes (Macfarlane et al., 2007). Commercially available inulin type prebiotics contain a mixture of these varying fructans; an

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example is oligofructose-enriched inulin (enrichment/mixture of inulin with oligofructose) (Gibson et al., 1995; Kleesen et al., 1997; Tuohy et al., 2001; Ping Su et al., 2007; Fritzen-Freire et al., 2012).

1.3.1 Synbiotics

The combination of probiotics and prebiotics termed synbiotics has been applied to improve the survival and viability of the probiotics in food products to provide health benefits to individuals (Gallaher and Khil, 1999; Sultana et al., 2000; Mortazavian et al., 2007; Kalliomäki, 2009; Sekhon and Jairath, 2010; Kolidas and Gibson, 2011). This is so because the prebiotics act as substrates for probiotics, thus leading to an increased number of commensal bacteria in the gut (Fooks and Gibson, 2002). Synbiotic combinations include (*Lactobacillus* GG and inulin), (*Bifidobacterium* and FOS), (*Lactobacillus acidophilus* and inulin), (*Lactobacillus*, FOS and inulin) (Fooks and Gibson, 2002). Products which contain synbiotics include Probio'stick (Probiocap), yoghurt, nutrient and chocolate bars (Attune, USA) and capsules Geneflora™ (Burgain et al., 2011). It has been reported that improved lipid metabolism and digestion are attributed to the use of products containing synbiotics (Sekhon and Jairath, 2010). A study conducted by Liong et al. (2007), reported decreased levels of total plasma, cholesterol, triacylglycerols and LDL levels in 24 hyperchloesterolemic male pigs at the eight week after ingesting synbiotic formulations containing *L. acidophilus* ATCC 4962, FOS, mannitol and inulin.

Though the aforementioned methods and formulas have helped to improve probiotic viability, studies report that maintaining the viability and stability of probiotics is still a major challenge for food industries (Mattila-Sandholm et al., 2002). However, processes which provide suitable barriers as a means of shielding the probiotic strains from stressful conditions as well as preserving their viability have been implemented. These processes include embedding the cells in immobilised biofilm, as well as the use of microencapsulation technique (Desmond et al., 2002; Picot and Lacroix, 2004; Thantsha et al., 2009).

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1.4 Microencapsulation

1.4.1 Overview

Microencapsulation is a method which entraps or packages an active material (solid, liquid, gas and living cells) in a wall material (hydrocolloids) in order to decrease the loss of viable cells by protecting them from adverse effects (Anal and Singh, 2007; Heidebach et al., 2012). These adverse effects include oxygen, certain solvents, high temperatures, pH, moisture, bacteriophage attack, negative effects of freeze-drying and freezing (Krasaekoopt et al., 2003; Burgain et al., 2011). Studies have reported the ability of microencapsulation to actively coat and protect probiotic strains, control oxidative reactions, extend shelf life, improve the release of strains at controlled rates and at specific times, shroud odours and colours and mask flavours in food industries (Chen and Chen, 2007; Rokka and Rantamäki, 2010). Microencapsulation allows for the production of microcapsules with small diameters that do not negatively affect the organoleptic qualities of food products into which they are incorporated (Burgain et al., 2011). The diameter of microcapsules obtained range from micrometres to millimetres and they encourage uniform cell growth while reducing mass transfer limitations (Dembczynski and Jankowski, 2002; Franjione and Vasishtha, 1995; Jankowski et al., 1997; Gibbs et al., 1999). Release of the core from the matrix or coating material occurs either by mechanical rupture of the cell wall, fracture by heat, change in pH or temperature, solvation, or diffusion through the wall (Franjione and Vasishtha, 1995; Gibbs et al., 1999). It is important to note that the coats used for microencapsulation must be GRAS approved by the FDA (Food and Drug Administration). The coating materials must stabilize the active material, not negatively affect the active ingredient and should release active material at target site (Vasishtha, 2003). However, successful microencapsulation is highly dependent on the strain used, coating material used and the factors involved in a specific microencapsulation method (Vasiljevic and Shah, 2008; Burgain et al., 2011). Various methods of microencapsulation exist, which have yielded varied results with regards to their ability to protect probiotics.

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1.4.2 Methods of microencapsulation

The methods of microencapsulation are grouped either as physical or chemical (Anal and Singh, 2007; Vidhyalakshmi et al., 2009). The chemical methods include interfacial polymerisation, in situ polymerisation and matrix polymerisation) (Vidhyalakshmi et al., 2009). The physical methods include extrusion (droplet method), emulsion (two step method), pan coating, fluidized bed coating, spray drying, spray coating, spray chilling, freeze drying and a novel microencapsulation under supercritical carbon dioxide (Moolman, et al., 2006; Thantsha et al., 2009; Heidebach et al., 2012). It is crucial that an appropriate method of microencapsulation be selected, depending on the substance to be encapsulated. Given that each method produces a characteristic microcapsule as well as its own limitations and advantages with regards to delivering the probiotic strains to the targeted area (Prakash et al., 2011). The microencapsulation techniques commonly used are explained in detail below.

1.4.2.1 Extrusion

Extrusion involves mixing probiotic cells with a hydrocolloid (e.g. alginate) and then extruding the mixture through a nozzle at a high pressure (droplet forms) into a hardening solution (e.g. calcium chloride). Extrusion produces gel beads ranging from 2 to 4 mm. The bead sizes obtained depends on the diameter of the nozzle and the viscosity of the cell-hydrocolloid mixture (Rokka and Rantamäki, 2010). This method is cost effective, requires little labour, produces relatively high retention of viable cells and requires no deleterious solvents. Lactobacilli and bifidobacteria have been encapsulated by this method with other materials like whey proteins and xanthan-gellan mixture (Groboillot et al., 1994; Rokka and Rantamäki, 2010).

1.4.2.2 Emulsion

Emulsion was first introduced by Nilsson et al. (1983) and it involves entrapping probiotic cells by a water/oil emulsion (Sheu and Marshall, 1993). This method requires the mixture of the discontinuous phase (probiotic cell and hydrocolloid) and the continuous phase (any vegetable oil). This mixture is homogenised to form the water/oil emulsion, to which an

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emulsifier (Tween 80) is added to improve homogeneity. Calcium chloride is added to break the emulsion, thereby producing instant gelation while stirring. The microcapsules obtained are collected by centrifugation, and they range from 20 µm to 2 mm (Heidebach et al., 2012). Studies have shown that the capsule size can be controlled by adjusting the speed of the magnetic stirrer and maintaining the viscosity ratio between the vegetable oil and alginate (Heidebach et al., 2012; Krasaekoopt et al., 2003). Lactobacilli have been encapsulated in calcium alginate gels using this method (Rokka and Rantamäki, 2010).

1.4.2.3 Spray drying

Spray drying is most commonly used in food industries and it involves converting liquid substances to dried powders. Spray drying was first utilised in the 1930's to encapsulate flavours, produce coffee extracts and detergents (Peighambardoust et al., 2011; Chávarri et al., 2012). It is utilised as a long term preservation technique for probiotic cultures, it is highly energy efficient and offers high productivity at low costs which ensures easy industrial applications (Petrovic et al., 2007; Peighambardoust et al., 2011). However, the use of spray drying for encapsulating probiotics for food application is still a challenge as a result of the low survival rate of the probiotic strains witnessed during drying (Rokka and Rantamäki, 2010). This method exposes the bacterial cells to high temperatures as well as dehydration step which lead to DNA and cytoplasmic membrane damage (Petrovic et al., 2007). The high temperature utilised in this method limits its use on bifidobacteria species (Picot and Lacroix, 2004). Inactivation of bacterial cells by temperature could be due to loss of genetic material. Inactivation by dehydration occurs as a result of drying which removes water from cell components (Peighambardoust et al., 2011). Water is necessary for maintaining various cell components such as lipid bilayer and cytoplasmic membrane which are the most sensitive and thus are targets for dehydration induced damage (Riveros et al., 2009).

The basic steps of spray drying are preparation of aqueous solution, homogenisation, atomization and dehydration of the atomized particles (Burgain et al., 2011). Preparing the aqueous solution involves dispersing core material into a polymer (solvent + shell material). The aqueous solution is homogenised by applying pressure. At this point, the core material is captured inside the wall material forming a stable emulsion. This is followed by atomization, which is a very critical step during spray drying because the liquid is atomized into droplets

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by bringing them in contact with hot air in a drying chamber (Peighambardoust et al., 2011). Depending on the type of spray dryer used, the solvent is then evaporated based on the air flow pattern (Co-current, counter current or mixed flow) (Peighambardoust et al., 2011; Chávarri et al., 2012). However, most spray dryers are designed to use the co-current air flow because this increases rapid surface evaporation and it is safe for heat sensitive materials (Chávez and Ledeboer, 2007; Peighambardoust et al., 2011; Chávarri et al., 2012). After evaporation, the dried sample is passed to the cyclone powder collector and retrieved in the collection vessel (Burgain et al., 2011). The limitations to using spray drying have been overcome by optimising and maintaining a low outlet air temperature which is the main reason for viability loss in cells (Picot and Lacroix, 2004).

1.4.2.4 Freeze drying

Freeze drying, like spray drying, is used for the long term preservation of various probiotic cultures. It is used to preserve immobilised and non-immobilised strains (Petrovic et al., 2007). Freeze drying involves freezing the culture, followed by the removal of water by sublimation under high vacuum (Carvalho et al., 2004). It results in the formation of ice crystals, as well as an increased salt concentration within and around the cells. This causes damage to the cell structure as a result of changes in fatty acid and protein structure (Chen et al., 2006). In order to prevent cell damage, compounds known as cryoprotectants have been utilised. These cryoprotectants include skim milk, glucose, glycerol, lactose, trehalose and inositol (Capela et al., 2006). Cryoprotectants associated with protecting cells during desiccation are grouped into three categories. These include protectants that penetrate the cell wall and membrane (glycerol), those that penetrate cell wall but not the membrane (amino acids, disaccharides and oligosaccharides) and those that do not penetrate the cell wall nor the membrane (polysaccharides and proteins) (Carvalho et al., 2004). Studies have reported that these compounds lower the transition temperature of the dried membranes by replacing water between lipid head groups, which in turn prevents the leakage of intracellular substances from cells (Chen et al., 2006). Disaccharides (lactose and sucrose) inhibit free radicals which cause viability loss when lyophilised bacterial cells are exposed to air, while the amino groups of amino acids (cysteine) react with the carboxyl group of probiotic strains, thus stabilizing the protein structures (Champagne et al., 1991).

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It is important to note that though cryoprotectants have been used to protect cells, research has also focused on the use of emulsions to improve the protection of probiotic strains during freeze drying. Dianawati et al. (2013) reported the use of emulsions containing various cryoprotectants for the protection of *Bifidobacterium longum* 1941. Significant survival rates have been reported for *Lactobacillus paracasei* subsp. *paracasei* suspended in emulsions before freeze drying (Heidebach et al., 2010). The method of emulsion requires a stable water/oil system and depending on the encapsulating material and drying solvent used, protection of probiotic strains can be achieved (Rokka and Rantamäki, 2010). However, the use of solvents like dichloromethane is less explored. Dichloromethane (DCM) is widely used as an extractant in food and pharmaceutical industries (Fouad et al., 2011; Miller and Gil, 2012). Dichloromethane is a suitable solvent for this study because it lacks oxygen atoms, has good solubilisation abilities, it is highly volatile, non-flammable, can dissolve various materials, has a low boiling point and enthalpy of vaporization of 39.8°C and 28 kJ/mol respectively (Methylene chloride EHC 164, 1996; Olvera-Bello et al., 2010). It has rarely been used in the microencapsulation of probiotics, possibly as a result of its toxicity to both human and bacteria (Miller and Gil, 2012). With regards to its toxicity in humans, DCM has been grouped with the group II solvents by the International Conference on Harmonization (ICH). Group II solvents are considered less harmful with an approved exposure of 6 mg/day; hence exposure must be reduced to avoid toxic effects (Miller and Gil, 2012). With regards to its toxicity to bacterial cells, studies have reported that certain bacterial strains such as *Dehalobacterium formicoaceticum* (strictly anaerobic bacteria) have the ability to degrade and utilize DCM as a source of carbon and energy (Mägli et al., 1996). Mägli et al. (1996) reported that DCM is one of the chlorinated aliphatic hydrocarbons known to support the growth of strictly anaerobic bacteria. Looking at these reports, the possibility that DCM may not be detrimental to bifidobacteria, which are also a strict anaerobe; as well of its ability to solubilize the lipid based coating material used in the current study is worth investigating.

1.4.2.5 Spray coating

Spray coating method involves spraying of a liquid coating material over a core material (usually solid), which is already kept in motion in a vessel by the injection of air. This leads to solidification and formation of a layer at the surface. The properties of the coating material

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can be influenced depending on the direction or angle from which the material was injected (Champagne and Fustier, 2007). Spray coating has been used for the encapsulation and protection of probiotics during exposure to stressful environments (Gouin, 2004; Doleyres and Lacroix, 2005). Spray coating is easy to scale up and it has been adapted to allow for multiple layers of coating for improved probiotic protection. However, there is always a risk of increased cost as it is a technique in need of skilled personnel (Burgain et al., 2011).

1.4.2.6 Spray chilling

Spray chilling, just like spray coating involves spraying a lipid based coating material onto a solid or dry core material. A cooling air temperature (10°C to 50°C) is used to improve solidification of the lipid coating. There have been limited reports on the use of spray chilling; however, it has been used for the encapsulation of heat sensitive materials such as probiotics (Gibbs et al., 1999). Improved protection was reported when *Lactobacillus acidophilus* and prebiotics (inulin and polydextrose) were encapsulated in a lipid microparticle by spray chilling (Okuro et al., 2013).

1.4.2.7 Encapsulation under supercritical carbondioxide

Encapsulation of probiotics using supercritical fluids is a novel technique which has been recently introduced in food and pharmaceutical industries because it presents better advantages than the commonly used methods. This method of encapsulation is well suited for the encapsulation of probiotic strains because it does not expose the strains to high temperature, oxygen, water or any other solvents (Moolman et al., 2006). Above certain critical temperatures and pressures, supercritical fluids act like gases and compressible liquids which take the shape of their container (Demirbas, 2001). Hence, they have both gaseous and liquid like properties, with densities of 0.1 to 1 g/ml and lower viscosity and surface tension than solvents (Moshashaee et al., 2000; Demirbas, 2001). Compounds such as carbondioxide, acetone and propane have been utilised as fluids in supercritical application processes. The most utilised compound in pharmaceutical and food applications is carbon dioxide (Demirbas, 2001).

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Carbon dioxide is a good solvent, inexpensive, non-toxic, easy to remove from products and easy to recycle. It also has relatively low critical temperature and pressure of 31°C and 73 bar respectively (Hénon et al., 1999). Supercritical carbon dioxide ($ScCO_2$) is produced when CO_2 is compressed at a high atmospheric pressure (Hsieh and Ofori, 2007). The nonpolar solvent nature of supercritical CO_2 limits its use in dissolving polar molecules (Sarrade et al., 2003). This method of encapsulation only process polymers with low molar masses, which are associated with poor barrier qualities thus making them unable to protect sensitive core materials. This drawback was counteracted when an interpolymer complex was formed using Poly (vinyl pyrrolidone) (PVP) and Poly (Vinylacetate-co-crotonic acid (PVAc-CA), which led to improved barrier qualities (Moolman et al., 2006) Supercritical carbondioxide has been used for the successful encapsulation of *B. lactis* Bb12, *B. longum* Bb-46 and indomethacin (Moolman et al., 2006; Thantsha et al., 2009).

1.4.3 Materials used for probiotic encapsulation

Successful microencapsulation is based on the type of material or polymer used. The specific microparticle or capsule formed depends on the type of material used and this will also determine how the core materials are released. The encapsulating materials utilised must be approved by the FDA, have a GRAS status for food applications, possess good mechanical strength and show good compatibility with the core material (Reineccius, 2004). These encapsulating materials can function as shell materials (shields active materials from environmental stresses), emulsifiers and food additives (Corcoran et al., 2004; Burgain et al., 2011). Some encapsulating materials commonly used are discussed below.

1.4.3.1 Alginate

Alginate is a polysaccharide naturally derived from algae. It is composed of β-D-mannuronic and α-L-guluronic acids. Alginate has been used extensively in microencapsulation processes because it is not toxic and has been accepted as a food additive (Sheu and Marshall, 1993; Anal et al., 2003). The most accepted of the alginate hydrogels is the calcium alginate combination because it is inexpensive, as well as non-toxic. This combination forms an interior gelation that allows the immobilisation or entrapment of probiotic cells (Hansen et al., 2002). Various researchers have reported the utilization of calcium alginate to

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microencapsulate *Lactobacillus* and *Bifidobacterium* spp (Sultana et al., 2000; Dembczynski and Jankowski, 2002; Chandramouli et al., 2004). It has been observed that cell viability depends on the size of microcapsule and gel concentration used. Chang and Zhang (2002) coated *L. acidophilus* in a mixture of sodium alginate and hydroxypropyl cellulose at a ratio of 9:1. Results showed that the strains were not released in the colon as expected, due to sensitivity of alginate to acidic environments (Chang and Zhang, 2002). Also, in most cases, the microcapsules obtained had pores on their surfaces which are not desired for the protection of probiotic strains from external stresses (Burgain et al., 2011). Nonetheless, these limitations can be overcome by adding different additives or compounds like starch to improve protection of probiotic strains (Sun and Griffiths, 2000).

1.4.3.2 K- carrageenan

Carrageenan is a polymer which is naturally extracted from marine red algae. This polymer is made up of repeating D-galactose-4- sulphate and 3, 6- anhydro-D-galactose units (Rokka and Rantamäki, 2010). These units are connected by alternating α1-3 and β1-4 glycosidic linkages (Rokka and Rantamäki, 2010). K-Carrageenan serves as a gelling agent and a food additive. In order to use this material for probiotic cell encapsulation, a temperature of about 40°C – 50°C is required, at which cells are added to the polymer mixture (Chávarri et al., 2012). Gelation occurs after cooling of the mixture. The microcapsules obtained are stabilised by addition of potassium ions (Anal and Singh, 2007). Although the beads or microcapsules obtained by this material maintains the bacterial cells in a viable state, reports have shown that the beads produced are weak and cannot withstand stressful conditions (Chen and Chen, 2007) Studies have shown that encapsulation of both *B. bifidum* and *B. infantis* in k-carrageenan improved their survival in frozen ice milk which was stored for 10 weeks at -20°C (Kailasapathy, 2002). Audet et al. (1991) also showed that a combination of locust bean gum and k-carrageenan (2:1) improved survival of *Lactobacillus* because the gel beads were strengthened.

1.4.3.3 Starch

Starch consists of glucose units joined together by glycosidic bonds. Starch is mainly composed of amylose (linear glucose polymer joined by α-1-4 glycosidic bonds) and

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amylopectin (branched glucose polymer joined by α-1-4 and α-1-6 glycosidic bonds (Burgain et al., 2011). Probiotics like bifidobacteria can adhere to starch granules; as such they can be used as a vehicle for delivery of viable probiotics into the colon (Rokka and Rantamäki, 2010). A type of starch known as resistant starch also has prebiotic functions and can deliver probiotics into the colon, since it is not digested in the small intestine (Anal and Singh, 2007).

1.4.3.4 Cellulose acetate phthalate

Cellulose acetate phthalate is a polymer derived from cellulose and it has been used for the encapsulation and controlled release of drugs (Mortazavian et al., 2008). Cellulose acetate phthalate can be used for the encapsulation and protection of probiotics during exposure to simulated gastrointestinal conditions, as it does not disintegrate at acidic pH but becomes more soluble at higher pH (Fávaro-Trindade and Grosso, 2002; Burgain et al., 2013).

1.4.3.5 Gellan gum and Xanthan gum

Gellan gum is a polysaccharide derived from *Pseudomonas elodea*, which consists of repeating units of either glucose, glucuronic acid and rhamnose, joined together by α-1-3 glycosidic bond (Chen and Chen, 2007). Xanthan gum is derived from *Xanthomonas campestris* and consists of glucose, mannose and glucuronic acid (Sun and Griffiths, 2000). A combination of gellan and xanthan gum has been used for the encapsulation of probiotics because they do not easily disintegrate in acidic conditions (Sultana et al., 2000).

1.4.3.5 Chitosan

Chitosan is mainly composed of glucosamine units which can polymerize by the means of cross linking in the presence of anions and polyanions (Burgain et al., 2011). Chitosan is preferably used as a strengthening agent rather than an actual coat because on its own, the protection of probiotic is greatly reduced. Studies show that the encapsulation of probiotics with alginate and chitosan provided better protection in simulated gastrointestinal conditions (Chávarri et al., 2010).

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1.4.3.6 Gelatin and Milk proteins

Gelatin is a protein gum derived by partial hydrolysis of collagen, which sets to an irreversible gel when cooled (Rokka and Rantamäki, 2010). Its versatile properties allow for its combination with other encapsulating materials such as gellan gum, for the encapsulation of probiotics (Anal and Singh, 2007). Milk proteins have been used as a delivery system for probiotics in food industries, due to their natural properties. They are divided into caseins and whey proteins, of which the latter is more heat sensitive and thus affects the stability of emulsions (Picot and Lacroix, 2004).

The above mentioned materials are either derived from seaweed, plants, bacteria or animal (Rokka and Rantamäki, 2010).

1.4.3.7 Lipid based coating materials

Notably, the use of lipid materials for microencapsulation is yet to be fully investigated, possibly because of their inability to dissolve in polar solvents (Gouin, 2004; Heidebach et al., 2012). Studies have shown that encapsulation of probiotics using lipids has been mostly achieved by mixing the cells with molten fat, followed by cooling. However, dispersing the cells in oil poses a great challenge and has often failed (Picot and Lacroix, 2004). In addition, premature melting at high temperatures may make separation problematic; hence the application has been limited to solid foods (Heidebach et al., 2012). Modler and Villa-Garcia (1993) reported unsuccessful protection of probiotics encapsulated with butterfat during storage in frozen yoghurt. However, probiotics encapsulated in cocoa butter were slightly protected during storage in fermented and non fermented oat drinks (Lahtinen et al., 2007). Lipids such as compritol ATO 888, gelucire, precirol ATO 5 and stearic acid all have a GRAS (generally regarded as safe) status. They have recently been applied for the encapsulation and release of drug as they are water insoluble and non swellable, thus they can protect active agents during exposure to stressful environments (Özyazici et al., 2006; Patel et al., 2009; Gowda et al., 2010; Fouad et al., 2011). The use of these materials is discussed briefly below.

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1.4.3.7.1 Compritol

Compritol is a waxy material which belongs to the group glyceryl behenate (GBH) (glycerol ester bonded to behenate groups). It comprises of a mixture of mono (18%), di (52%) and triglycerides (28%) of behenic acid (Li et al., 2006). Compritol is highly hydrophobic (Hydrophillic-Lipophillic balance of 2) and has a melting temperature between 69°C and 74°C (Fini et al., 2011). It was initially used as a lubricant for tablets (Li et al., 2006; Sutanata et al., 1995), but recent applications such as direct compression and hot melt coating have utilised compritol as an important excipient used for sustained release of drugs (Barthelemy et al., 1999). Studies showed that the combination of Compritol 888 ATO with pectin (polysaccharide) was successful as colon targeted drug delivery system (Patel et al., 2009).

1.4.3.7.2 Carnauba wax

Carnauba is a waxy material which comprises of fatty alcohols (10%-16%), hydrocarbons (1%-3%) and a mixture of fatty acid esters (80%-85%). It has a melting temperature between 82°C - 86°C (Fini et al., 2011; Özyacizi et al., 2006). Carnauba is a non-toxic substance, which has also been reported to be hypoallergenic in nature. Carnauba is only broken down by certain solvents but is however water impenetrable, which indicates that its durability is able to create an environment devoid of stress for an active agent (Fini et al., 2011).

1.4.3.7.3 Gelucire and Vegetal

Gelucire comprises of a mixture of glyceryl mono-, di- and tristearates. Gelucires are inert and waxy substances used as excipients for sustained and controlled release of drugs into the colon (Moes, 1993; Sutanata et al., 1995). They have low melting temperatures (from 33°C to 65°C) and Hydrophillic-Lipophillic balance (HLB) values ranging from 1-18 (Sheu and Hsia, 2001). This indicates that those with low HLB can delay the release of compounds and those with high HLB can quickly release the compounds (Sheu and Hsia, 2001). Gelucires have been commonly used for drug delivery because they are biocompatible, biodegradable, no solvents required and do not show gastric irritation (Porter and Charman, 2001).

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Vegetal is a glyceryl distearate produced from vegetables with melting temperatures between 53°C-58°C. It comprises of mono, di and triglyceride contents of (33%, 37% and 30% respectively) (Gattefossé, technical and material data sheets, 2010). Vegetal can be used as a dietary supplement and for oral drug delivery (as a taste masking agent and a modified release agent) and thus is certified safe for human consumption. It is also a non polar fatty acid which is also insoluble in water but can be dissolved by solvents like dichloromethane and ethanol (Gattefossé, technical and material data sheets, 2010).

1.5 Food products containing encapsulated probiotics

The production and introduction of encapsulated probiotics into various food products has become popular since consumers desire functional foods that will provide health benefits to them (Mortazavian et al., 2007). Encapsulated probiotics have been introduced into foods such as mayonnaise, chocolate, sausages, biscuits, fruit juices and most commonly, dairy products (Khalil and Mansour, 1998; Ainsley et al., 2007; Ding and Shah, 2008; Allgeyer et al., 2010; Possemiers et al., 2010). Probiotics are sensitive to heat and thus are incorporated into dairy products which are stored under refrigeration. Dairy products are healthy, easily accessible and quick to consume (Allgeyer et al., 2010; Burgain et al., 2011). The most common dairy products are briefly discussed below.

1.5.1 Frozen ice cream

The high acidity of the product and freeze injury encountered in ice cream has made incorporation of probiotics very challenging. However, encapsulation of probiotics has so far averted these challenges (Chen and Chen, 2007). Studies have shown that encapsulation of probiotics not only enhanced viability in ice cream but had no negative impact on the sensory quality of the product (Godward and Kailasapathy, 2003b). Homayouni et al. (2008) also demonstrated that the high amount of total solid found in ice cream and the addition of prebiotic further improved viability of probiotics.

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1.5.2 Cheese

It has been reported that cheese, especially cheddar has the potential to carry and protect probiotics from acidic stresses due to its high fat content (Stanton et al., 1998). Studies have reported that microencapsulation is not essential for increasing viability of probiotics in cheddar cheese (Kailasapathy, 2002). However, in the case of fresh cheese, it is necessary to encapsulate probiotics before incorporation, due to the low pH of the product (Godward and Kailasapathy, 2003a; Kailasapathy, 2002). Studies have shown that encapsulation of bifidobacteria before incorporation into cheese, not only protected the cells, but also did not negatively affect the sensorial properties of the cheese (Dinakar and Mistry, 1994; Stanton et al., 1998; Özer et al., 2009).

1.5.3 Yoghurt

Of the dairy products ingested for health benefits, yoghurt is the most common, because its sensorial qualities have the most effect on consumers (Shah, 2000). Yoghurt is known to come from the word jugurt (Turkish) and is consumed worldwide (Tamime and Robinson, 1999). Yoghurt is made from raw milk extracted from cows, goats and sheep. Since raw milk has its own natural lactic acid bacteria, it is important that it is free from air contaminants or antimicrobials, as they would inhibit the bacterial cultures (Lee and Lucey, 2010). Inhibition of the bacterial cultures can lead to unfavourable tastes, subsequent rejection by the consumers and ultimately cause economic losses. The beneficial effects of yoghurt have made it very popular in various countries of the world, of which it is given different names (Chen and Chen, 2007). There are two major types of yoghurt and they include set and stirred style yoghurts. Both types of yoghurt are produced through the same processing steps (Figure 1.2) (Lee and Lucey, 2010). The only difference lies in the fact that set yoghurt forms a continuous gel structure during fermentation while the gel formed in stirred yoghurt is disrupted by agitation following fermentation and cooling (Tamime and Robinson, 1999).

With the increased awareness of yoghurt came the need for regulations which state, what should be in yoghurt and how it must be treated (USDA, 2001). All food additives added to yoghurt must have a GRAS status; yoghurt must not be bitter, rancid or unpleasant, must have a homogenous, smooth and firm texture (USDA, 2001). In 2002, the Food and

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Agriculture Organization (FAO) set standards which demand that yoghurt must be a product of fermentation by *Lactobacillus delbrueckii spp bulgaricus* and *Streptococcus thermophilus*, and microorganism claim must specify at least 10^6 cfu/g.

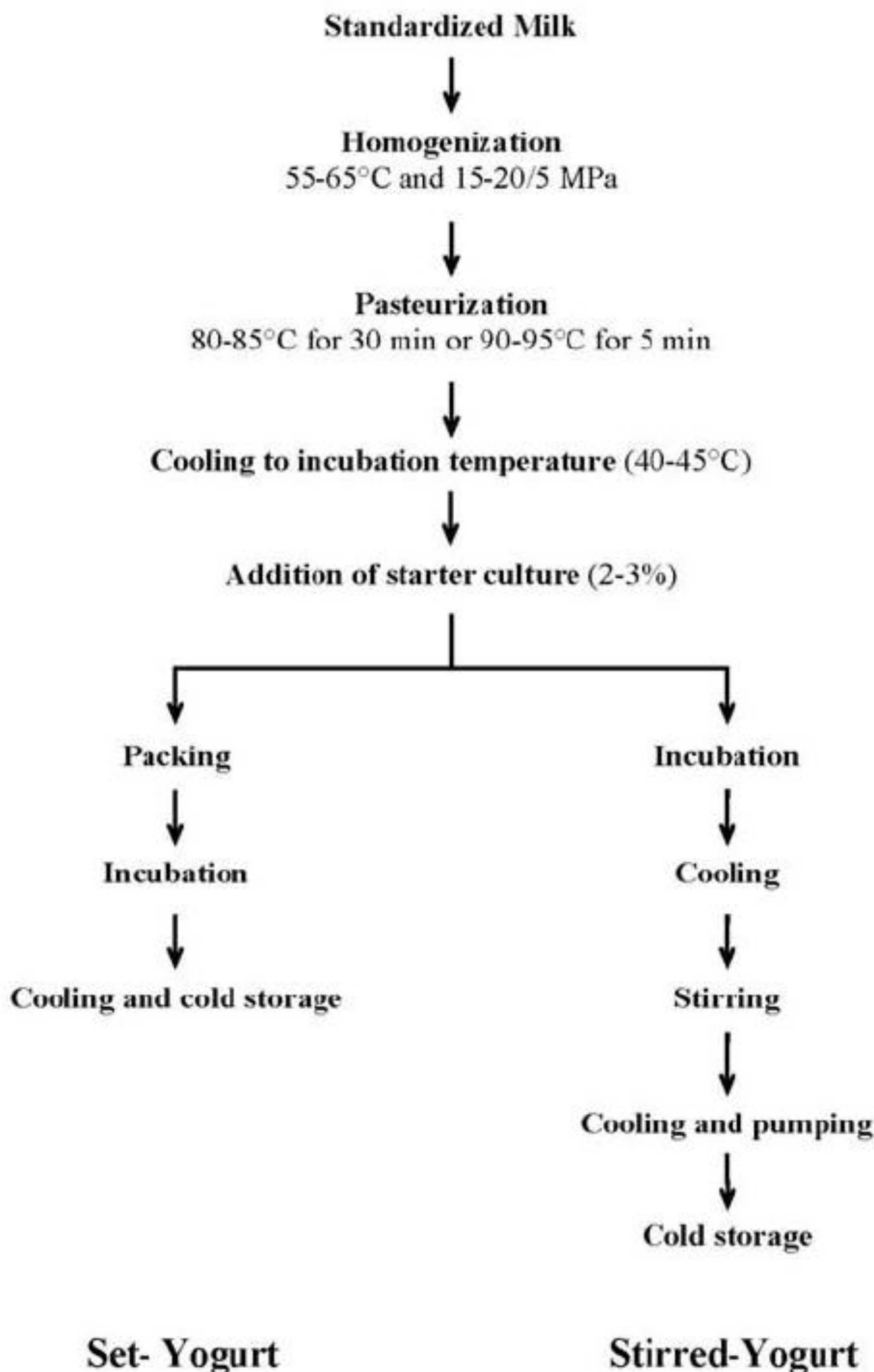

Figure 1.2: Processing steps for the production of set and stirred style yoghurts (Lee and Lucey, 2010).

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The desire to make yoghurt an accessible but functional food product, led to the introduction of probiotics. Unfortunately, studies have reported that the probiotic strains do not survive in yoghurt (Heller, 2001). This is due to factors which include the low pH level (4.2 - 4.6), acid produced during refrigeration storage and antimicrobial substances produced by the starter cultures (Shah, 2000; Heller, 2001). In an attempt to reduce the factors mentioned above, a two-step fermentation procedure was introduced. This involved an initial fermentation by probiotics, then fermentation by starter cultures, as this improved probiotic survival (Lankaputhra and Shah, 1997). In order to improve probiotic survival in yoghurt, other researchers found microencapsulation to be more effective (Sun and Griffiths, 2000; Picot and Lacroix, 2004). Furthermore, the encapsulation of probiotics with prebiotics became an even more promising method for probiotic improvement (Capela et al., 2006; Burgain et al., 2011). Addition of prebiotics is known to improve the sensorial qualities of yoghurt as they are carbohydrate derived fat replacers (Surisuvor et al., 2013). Since yoghurt is known for its therapeutic and nutritional values, it is important that the probiotic cultures present are properly quantified and its sensorial properties evaluated, before it is released into the market (Hootman, 1992; Gonzalez et al., 2011). With regards to post acidification during storage of yoghurt, studies have reported that the addition of probiotics slows down post acidification (Kailasapathy et al., 2008).

1.6 Methods for quantification of probiotics in foods

Maintaining the shelf life of probiotic food products is very essential; as this will enable the provision of their health benefits to consumers. Shelf life is the length of time in which the quality of food product is considered fit for consumer consumption. Shelf life maintenance requires; quantification of viable probiotics in the food products and the sensorial evaluation of the foods to which these probiotics are incorporated.

An improved assessment of probiotics in foods requires the use of detection methods which are cost effective, easy to perform and have higher sensitivity (lower detection limits). There are various methods of analysing and quantifying probiotics cultures and this will depend on the type of strain to be quantified, the enumeration method used, the technology and equipment and the objective of the experiment. Since various strains have specific

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characteristics, metabolic activities and growth parameters, they may require a combination of the methods, some of which will be briefly discussed.

1.6.1 Conventional method

Plate count method has been used as a traditional way of determining and estimating the number of viable probiotic cultures by means of culturing on selective media (O' Sullivan, 1999; Auty et al., 2001; Lahtinen et al., 2006). Variations of plate counting include spread and pour plating techniques, of which the latter limits the exposure of sensitive cells to oxygen, thus improving their growth (Collee et al., 1971; O' Sullivan, 1999; Madigan and Martinko, 2006). Plate counting is still the most common method used because it is cost effective, easily accessible and allows for biochemical and physiological analyses of bacterial cells (Lahtinen et al., 2006). However, it is a laborious, time consuming method that requires long incubation times. It is also associated with clumping of bacterial cells, and only counts live cells capable of growing on a selective medium, thus dormant cells remain undetected, which lead to underestimation of numbers of bacterial cells (O' Sullivan, 1999; Auty et al., 2001; Lahtinen et al., 2006). Plate counting has been used in conjunction with certain biochemical and molecular methods which enable detection of dormant cells (Bunthoff and Abee, 2002; Macfarlane and Macfarlane, 2004; Lahtinen et al., 2006; Kramer et al., 2009; Rokka and Rantamäki, 2010). These methods include microscopy, flow cytometry, microplate fluorochrome assay, quantitative real time PCR, polymerase chain reaction (PCR) and fluorescent in situ hybridisation (FISH) (Maidek et al., 2001; Wagner et al., 2003; Ventura et al., 2003; Macfarlane and Macfarlane, 2004; Biggerstaff, 2006; Lahtinen et al., 2006; Garner et al., 2009; Sekhon and Jairath, 2010). Some of which will be briefly discussed.

1.6.2 Biochemical methods

1.6.2.1 Fluorescent microscopy

Fluorescent microscopy has been utilised over the years to study cell structures and their viability in relation to encapsulation for improved probiotic protection (Dykstra, 1992; Madigan and Martinko, 2006; Moolman et al., 2006; Allan-Wojtas et al., 2008). This method

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involves staining of the bacterial cells with fluorescent dyes, which is accompanied by a visual imaging of the stained cells by a fluorescent microscope such as the confocal scanning laser microscope (CSLM) (Auty et al., 2001; Hibbs, 2004a; Alakomi et al., 2005). Fluorescent stains such as Live/Dead BacLight™ kit enables the differentiation between live and dead bacterial cells (Lee et al., 2009). This kit contains two nucleic acid dyes, propidium iodide (red fluorescent dye) and SYTO 9 (green fluorescent dye). SYTO9 stains all cells irrespective of the condition of the cell membrane. When propidium iodide (PI) is used, it replaces SYTO9 and stains only cells with compromised membranes. Hence, loss of membrane integrity allows penetration of PI across the membrane to stain the nucleic acid red (Auty et al., 2001; Lahtinen et al., 2006). The Live/Dead bacterial kit has been used for analysing sensitive samples like probiotic cultures, as it permits dual staining of probiotic cells for accurate viability testing based on the condition of the cell membrane (Jepras et al., 1995; Lahtinen et al., 2006). In combination with this staining, live and dead bacteria cells have also been distinguished using flow cytometry and microplate fluorochrome assay (Alakomi et al., 2005).

1.6.2.2 Flow cytometry and microplate fluorochrome assay

Flow cytometry quantifies bacterial viability by separating bacteria according to their differences in membrane integrity. Membrane integrity differentiates live, dead and damaged cells by allowing or excluding the binding of fluorescent dyes to the nucleic acid (Auty et al., 2001; Paparella et al., 2012). The principle of flow cytometry involves analysing particles flowing in a single file in a stream of fluid while being intercepted by light source (usually arc lamps and lasers). The light source is reflected as a narrow beam of light at specific wave lengths, which are filtered and transmitted into photomultiplier tubes to detect various fluorescent dyes (Bunthof et al., 2001). Using light scattering, excitation and emission of the fluoresced molecules, thousands of cells are analysed per second and multiparameter data is obtained (Bunthof et al., 2001). The light scattered by cells during flow cytometry analysis are reported as forward scatter (relates to cell size) and side scatter (relates to internal structure of cells/ granularity of particles) (Veal et al., 2000). Fluorescent dyes can be utilised for membrane integrity assessment (PI and ethidium bromide) or for viability assessment {carboxyfluorescein diacetate (cFDA), TOTO series (TOTO 1) and SYTOX series (SYTO9)} (Rault et al., 2007; Paparella et al., 2008). These fluorescent dyes have

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been used singly or in combination to estimate physiological states of cells such as membrane integrity, cell respiration and metabolic activity (Veal et al., 2000; Auty et al., 2001; Münoz et al., 2009). The advantages of using flow cytometry are that large number of cells are analysed individually and at a fast rate. However, the flow cytometer is very complex and it requires that samples are clean and must not contain interfering particles which limit accuracy. Furthermore, operating the instrument requires personnel with an adequate understanding of the principles and operating system of the machine in order to interpret accurate results (Ormerod, 2000).

On the other hand, microplate fluorochrome assay has been used to replace laborious and extensive techniques while providing distinctions between live and dead cells (Alakomi et al., 2005; Masco et al., 2007). This technique allows a variety of experiments to be measured at the same time. This technique is used in conjunction with multiwell plates like the 96 well plates and it measures absorbance, fluorescence intensity and polarisation, luminescence and time resolved fluorescence. Light passes through an excitation filter, through a light guide to reach the plate well, and then the light is emitted back into another light guide and through an emission filter to the detector. A standard curve is often used to determine the value of the experimental samples (Masco et al., 2007).

In addition to quantifying probiotics in foods, one must also consider the possible changes which may occur in the food products over time during storage due to their presence. These changes might affect the organoleptic properties of the foods into which they are incorporated. Therefore, in order to preserve the quality of food products during storage, its sensory and physico-chemical characteristics must be evaluated.

1.7 Sensorial and physico-chemical evaluation of probiotic foods

Hootman (1992) explained sensorial evaluation as a method which measures the attributes of food products by using trained human subjects as well as instruments. Sensorial evaluation is a descriptive method which emerged in the early 1990's and has focused on analysing the flavour, texture, aroma, colour and general appearance of food products (Hootman, 1992). This descriptive method has been applied to foods such as yoghurt as a way of correlating its sensorial properties to consumer acceptance and preference (Harper et al., 1991; Rohm et al.,

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1994; Ott et al., 2000). The results obtained provide industries with an in-depth knowledge of what consumers would prefer. As already mentioned, this analysis requires human subjects or panellists whom are trained for months to act as scientific instruments to provide accurate and objective evaluations of various food samples. There are six steps to which panellists are trained and they include recruiting and screening potential panellists, organising an orientation session, conduct further screening sessions, developing descriptive terms and evaluation scales, determining the reproducibility and consistency of panellist rating and finally using panellists to evaluate samples (Lawless and Heymann, 1998). The descriptive terms used to define food properties are not definite, as they change in relation to how well the panellists utilise their five senses (taste, smell, touch, sound and sight) (Lawless and Heymann, 1998).

On the other hand, studies have also shown that descriptive analysis has been used to correlate instrumental measurements (physico-chemical analysis) to sensorial analysis performed by panellists (Ott et al., 2000). Food industries have developed interest in the use of instruments to analyse the micro and macrostructures of food (Mortazavian et al., 2009). Physico-chemical analysis provides a better understanding of the sensorial qualities of food in cases where the panellists fail to be consistent in their analysis. Two methods have been adopted for physico-chemical analysis. These methods include chemical and structural analysis (Benezech et al., 1994; Tunik, 2000; Mortazavian et al., 2009). For the purpose of this study, only these methods will be briefly discussed with focus on their uses in yoghurt.

1.7.1 Chemical Analysis

Chemical analysis focuses on flavour analysis of yoghurt (taste and aroma), milk constituents (carbohydrates, fats and proteins) and chemical assays such as enzyme activities. Various methods have been used to analyse the chemical components of yoghurt. These include among others gas chromatography, high performance liquid chromatography and electronic nose methodology (Tamime et al., 1996; Tamime and Robinson, 1999). These methods will be briefly discussed.

Gas Chromatography (GC) – This method involves a sampling and instrumental analytical step and has been used to identify and quantify volatile compounds found in yoghurt. Ott et

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al. (2000) utilised this method to identify 60 flavour compounds in yoghurt which include 1-nonen-3-one and methional. GC method was also used to study the effect of culture composition containing *L. delbrueckii ssp. bulgaricus*, *Streptococcus salivarius ssp. thermophilus* and *L. acidophilus* on the aroma profiles of fermented milks (Gardini et al., 1999).

High Performance liquid chromatography (HPLC) – This method has been used to determine the presence of organic acids (lactic, acetic, formic, pyruvic and butyric acid) in yoghurt (Adhikari et al., 2000). Using this method, total acids are separated on an ion-exchanger, followed by protein precipitation with trichloro-acetic acid, centrifugation and acid quantification (Lee et al., 2000). Bouzas et al. (1991) evaluated the effects of microencapsulated bifidobacteria on the concentration of lactic and acetic acids found in yoghurt using HPLC.

Electronic nose methodology (ENM) – This method is utilised in food industries for the analysis of flavour release profiles during fermentation in yoghurt. ENM is also used for insitu determinations and non-destructive sensing (Mortazavian et al., 2009).

1.7.2 Structural Analysis

Structural analysis focuses on textural and rheological analysis, and microstructural analysis. Textural and rheological analysis focuses on methods of evaluating gel and liquid properties of yoghurt. On the other hand, microstructural analysis focuses on microstructural images and assays (Mortazavian et al., 2009). For the purposes of this study, textural and rheological analysis will be further discussed.

When determining rheological properties (deformation and flow characteristics) of food products, factors to consider include firmness, elasticity and viscosity (Mortazavian et al., 2009). Viscosity measures the rate of flow of a liquid, to which they can be classified as being pseudoplastic (fluids that return to equilibrium immediately after stress), thixotropy (fluids which take time to equilibrate after stress), rheopectic (fluids whose viscosity increases with constant stress) and newtonian (fluids with the tendency to not flow easily) (Lee and Lucey, 2010). Yoghurt has been classified as a Newtonian fluid (highly viscous)

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whose viscosity lowers with constant stirring or agitation, hence is not a true thixotropy (Walstra et al., 1999). Methods used to analyse texture and rheological properties include:

Penetration and Texture Profile Analysis – The penetration test involves puncturing sample surface with a probe to measure consistency, hardness and strength of the gel structure. The texture test utilises double compression forces on samples to measure hardness, cohesiveness and mouthfeel (chewiness) of sample (Mortazavian and Sohrabvandi, 2004).

Viscometric tests – This method involves stirring the yoghurt with a glass rod to evaluate and assess the viscosity of the fractured gel. Two types of viscosities, namely elongation viscosity and shear viscosity, can be analysed (Mortazavian et al., 2009). Shear viscosity is mostly studied and commonly analysed with the rotational viscometers. Rotational viscometers are sensitive instruments with the ability to take accurate measurements of viscosity (Rohm, 1992). Viscometric tests measure from zero shear rates to high shear rates, hence if yoghurt is poured down an orifice with a known diameter and a fixed temperature, the distance and viscosity during a specific time can be measured (Cullen et al., 2000).

Oscillatory tests – This method involves measuring the viscoelastic properties of gel and other gel like materials. Sample materials are put through compression and shear stress before measurements are done (Mortazavian et al., 2009).

1.8 Conclusion

Probiotics provide several health benefits when ingested in sufficient amounts. However, probiotics are faced with the challenge of remaining viable throughout manufacturing processes, storage and during consumption. Microencapsulation is a method used to ensure that probiotics remain viable till they reach the target area. Though various methods of microencapsulation have been used, freeze drying is a method used for the long term preservation of probiotics. The encapsulating material plays a significant role on how best probiotics are protected. Thus, Vegetal BM 297 ATO in addition to inulin is to be explored as a suitable material for the encapsulation and protection of probiotics using freeze drying. The amount of viable cells encapsulated must be investigated, to ensure that they are in sufficient amounts before consumption. Thus, in this study plate counting will be used since it is still

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widely used for bacterial enumeration. However, plate count has been associated with bacterial underestimation which could provide unreliable results. Thus, rapid methods such as LIVE/DEAD *Bac*Light kit will also be used in conjunction with microplate fluorochrome assay and flow cytometry for viability analysis.

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CHAPTER 2

Production and characterization of freeze dried Vegetal BM 297 ATO-inulin lipid-based synbiotic microparticles containing *Bifidobacterium longum* LMG 13197

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2.1 Abstract

Microencapsulation as a method of protecting probiotic cells has been extensively researched. However the potential of lipid-based coating materials for the encapsulation of probiotics has not been extensively evaluated. This study aimed at the manufacturing and characterization of Vegetal BM 297 ATO-inulin-*Bifidobacterium longum* LMG 13197 microparticles produced by freeze drying. Emulsions containing 1%, 1.5%, 2%, 3.5% or 5% (w/v) inulin were prepared, with or without a centrifugation step before freeze drying. Morphological properties, particle size distribution and encapsulation efficiency of the resulting microparticles as well as their ability to preserve viability of the enclosed *B. longum* LMG 13197 cells were evaluated. The microparticles produced from both formulations were irregular, porous with concavities and contained high number of bacterial cells. High bacteria viability was obtained with non-centrifuged formulations containing 2% (w/v) inulin. Formulations with and without inulin had average particle sizes of 33.4 µm and 81.0 µm with encapsulation efficiencies of 82% and 88%, respectively. Thus, Vegetal-inulin microparticles produced by freeze drying have the potential to protect, deliver adequate amounts of probiotics and minimally affect the organoleptic properties of food products to which they will be added.

Keywords: Probiotics, *Bifidobacterium longum* LMG 13197, Inulin, Microencapsulation, Freeze drying, Vegetal BM 297 ATO.

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2.2 Introduction

There has been an increasing interest in probiotics as a health promoting tool due to the health benefits they provide (Sultana et al., 2000). These health benefits include relief from constipation, lactose intolerance and irritable bowel syndrome (Fooks and Gibson, 2002; Talwalkar and Kailasapathy, 2004; Vasiljevic and Shah, 2008). The strains which represent probiotics include, but are not limited to lactobacilli and bifidobacteria (Adhikari et al., 2000). Bifidobacteria strains are Gram positive, non-motile, rod shaped and anaerobic in nature. These bacteria are however, sensitive to light, moisture, oxidation and acidic environments. Hence, they do not remain viable during gastrointestinal transit and in certain food products (Adhikari et al., 2000). Probiotic strains would benefit from a physical barrier which can protect them from unfavourable environmental conditions. This in turn leads to an improvement in their survival during food processing and gastrointestinal transit (Brinques et al., 2011).

Microencapsulation has been successfully used by food industries to improve survival of probiotic strains (Sheu and Marshall, 1993; Doleyres and Lacroix, 2005; Appelquist et al., 2007; Chen and Chen, 2007; Pimentel-González et al., 2009; Thantsha et al., 2009; Chávarri et al., 2012). Microencapsulation entails entrapping or immobilising (coating) live microbial cells in capsules or matrices, which provide a physical barrier to harsh conditions and enables their sensitive bioactive contents/ living cells to be released at controlled rates and in specific environments in a viable/functional state (Champagne and Fustier, 2007; Rokka and Rantamäki, 2010). The overall objective of this procedure is to produce particle sizes ranging from 100 µm to 1 mm and extend shelf life of the probiotic cells (Champagne and Fustier, 2007; Petrovic et al., 2007). The microparticles within this size range have been produced by various methods of microencapsulation and are reported to not negatively affect the organoleptic properties of the foods into which they are incorporated (Anal and Singh, 2007; Zuidam and Shimoni, 2010; Burgain et al., 2011).

Freeze drying is a microencapsulation technique which has been used in combination with food matrices and cryoprotectants to protect probiotic bacteria (Chen et al., 2006; Meng et al., 2008; Pop et al., 2012). Studies have shown that in addition to damage to the membrane lipids and DNA of cells, freeze drying also affects the microparticle size and morphology of

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microparticles, thus altering their protective function towards probiotics (Champagne et al., 1991; Mortazavian et al., 2007; Meng et al., 2008). Precautions must be taken to reduce cell damage caused by the formation of ice crystals and high osmolarity, which occurs during freezing (Meng et al., 2008). Cryoprotectants such as skim milk, lactose, glucose and fructose have been used to suspend the cells before freeze drying (Chen et al., 2006). On the other hand, freeze drying is well suited for the preservation of probiotic cells because it avoids heat induced injuries to cells and it slows down most chemical reactions (Carvalho et al., 2004; Capela et al., 2006). Since this method occurs in a vacuum and in the absence of oxygen, the possibility of having an oxidative reaction is reduced (Carvalho et al., 2004).

Matrices which have been utilised for encapsulation include alginate, κ-carrageenan, chitosan and gellan gum) (Picot and Lacroix, 2004; Rokka and Rantamäki, 2010). Similarly, the addition of prebiotics such as inulin is another approach which has been used to improve protection of probiotics (Özer et al., 2005; Capela et al., 2006; Chen et al., 2006). Inulin is a prebiotic which resists hydrolysis by saliva and small intestine enzymes, thus has the potential to protect probiotics until they reach the colon (Gibson and Roberfroid, 1995; Corcoran et al., 2004; Özer et al., 2005; Akalin and Erisir, 2008; Peighambardoust et al., 2011; Fritzen-Freire et al., 2012). However, there are limited reports on the successful encapsulation of probiotics in lipid based coating materials. Therefore, the aim of this study was to manufacture and characterize the freeze dried Vegetal BM 297 ATO-inulin microparticles containing *Bifidobacterium longum* LMG 13197 based, on their morphology, particle size distribution, encapsulation efficiency and distribution of bacteria within the matrix.

2.3 Materials and Methods

2.3.1 Reagents and bacterial cultures

Biogapress Vegetal BM 297 ATO was obtained in powdered form from (Gattefossé SAS). *Bifidobacterium longum* LMG 13197 cultures were obtained from BCCM/LMG Culture collection (Belgium) as 20% glycerol stocks in MRS broth at -70°C and revived according to the manufacturer's specifications. Inulin (purity: 95%), polyvinyl alcohol (PVA) 87-89% partially hydrolysed (Mw: 13000-23000 Da), lactose monohydrated (purity: 99%) were

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obtained from Sigma Aldrich, South Africa, while dichloromethane (DCM) (analytical grade, purity: 99%) was obtained from Sigma Aldrich Laborchemikalien, Seelze.

2.3.2 Bacterial culturing and enumeration

Frozen stocks of *B. longum* LMG 13197 were allowed to thaw and then streaked out onto MRS agar plates and incubated in anaerobic jars with Anaerocult A gaspaks and Anaerocult C strip for 48 h at 37°C to obtain single colonies. A single colony was then inoculated into 5 ml MRS-cys-HCl broth and incubated anaerobically overnight at 37°C. One millilitre of the culture was then inoculated into 100 ml of MRS-cys-HCL broth and incubated anaerobically at 37°C overnight. This culture was subsequently subcultured into fresh 100 ml MRS-cys-HCL broth and grown as previously described. From the grown cultures, 1 ml was used for bacteria enumeration after dilution in Ringer's solution. A subsample of 100 µl was taken from the broth culture and suspended in 900 µl of ¼ strength Ringer's solution. A 10-fold serial dilution was performed and cells were plated out onto MRS-cys-HCL agar in triplicates and plates were incubated (General purpose incubator, Shel Lab) at 37°C for 72 h under anaerobic conditions. After incubation, number of viable cells were determined and recorded as cfu/ml.

2.3.3 Bacteria encapsulation

Encapsulation of bacteria was done according to the method used by Pimentel-Gonzalez et al. (2009) with modifications. One millilitre of *B. longum* LMG 13197 grown cultures was inoculated into each of the eight 250 ml flasks containing 100 ml of de Man-Rogosa-Sharpe (MRS) medium broth supplemented with 0.05% (w/v) of L-cysteine-HCL and incubated anaerobically at 37°C for 48 h. After incubation, the cultures were harvested by centrifugation, using the Eppendorf centrifuge 5804R (cooled to 4°C) at 20 800 g for 15 min. The pelleted cells were washed once with Ringer's solution and kept at 4°C for 5 min before encapsulation. A stable emulsion was prepared to allow for freeze drying. The first emulsion was prepared by suspending 0.7 g of bacteria (equivalent to 3.2×10^9 cfu/ml^{-1}) into 1 ml of a range of inulin solution 1%, 1.5%, 2%, 3.5% and 5% (w/v). The entire contents of each mixture were added to 1 ml of 2% (w/v) poly-vinyl-alcohol (PVA). The resulting 2 ml was finally added to 10 ml dichloromethane (DCM) containing Vegetal BM 297 ATO at a final

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concentration of 10% (w/v). These emulsions were homogenised (Silverson, L4R) at 8000 rpm for 5 min and left to stand at 25°C for 2 min. The second emulsions were prepared by mixing 15 ml of PVA 2% (w/v) and 5 ml of lactose 5% (w/v). The first emulsions were poured into the second emulsions and homogenised (Silverson, L4R, NIMR) at 8000 rpm for 5 min. The stable emulsions produced were allowed to stand in the fume hood for 5 h, to ensure evaporation of DCM.

After DCM evaporation, two formulations were produced from the stable emulsion. For preparation of formulation one (F1), the emulsion was frozen at – 20°C overnight and then freeze dried for 72 h in a Virtis bench top, SLC, freeze dryer at a condenser temperature and vacuum pressure of – 60°C and 0.26 millitor, respectively. For formulation two (F2), the emulsion was centrifuged at 10°C and 20 800 g for 10 min using an Eppendorf 5804R centrifuge. The pellet obtained was suspended in 6 ml of deionised water and then frozen at – 20°C overnight. This was then followed by freeze drying for 72 h as previously done for formulation one. The same protocol was used to prepare Vegetal BM 297 ATO microparticles encapsulating *B. longum* LMG 13197 without inulin. In this case, bacterial culture was suspended in 1 ml of deionised water. Control sample (unencapsulated cells) was prepared by resuspending *B. longum* LMG 13197 cells (approximately 3.2×10^{9} cfu/ml^{-1}) into 25 ml of sterile Ringer's solution and fast-frozen in liquid nitrogen for 5 min. The fast-frozen cells were then frozen at – 70ºC for 1 hr before freeze drying (Virtis bench top, SLC, freeze dryer) for 3 days at -75ºC. After freeze drying, all the samples were stored in tightly sealed sterile Schott bottles at 4ºC for further analysis.

2.3.4 Encapsulation efficiency

Encapsulation efficiency was determined by suspending 0.1 g of microparticles obtained from each formulation in 900 µl of DCM. Serial dilutions were performed using ¼ strength Ringer's solution and then 100 µl of each dilution was plated out onto MRS-cys-HCI agar in triplicates using the pour plate method. Plates were incubated at 37°C for 72 h under anaerobic conditions (Anaerobic jars with Anaerocult A gaspaks and Anaerocult C test strip). Encapsulation efficiency was calculated after colonies were counted using the equation below:

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$$EE\% = \frac{\text{(Total bacteria released from matrix)}}{\text{(Total bacteria before encapsulation)}} \times 100$$

2.3.5 Particle size analysis

Particle size distribution and mean particle size of the samples were analysed using a Malvern Mastersizer (Mastersizer MS3000, Malvern Instruments UK). The Mastersizer uses a laser diffraction technique which measures the intensity of light scattered as the laser beam passes through the samples which were dispersed in water. Triplicate samples of each dried formulation were measured at 25°C and their particle sizes were calculated.

2.3.6 Microscopy

2.3.6.1 Scanning electron microscopy

Microparticle morphology and bacterial distribution within microparticles was studied using a scanning electron microscope (SEM) according to the method of Qiang Zou et al. (2011). Freeze dried microparticles encapsulating bacteria were coated with carbon before gold sputtering under argon atmosphere (Emitech K550X, Ashford, UK). In addition, the microparticles were frozen in liquid nitrogen and ground using mortar and pestle. This enabled proper viewing of the bacterial distribution within the microparticles. The ground microparticles were transferred to aluminium pins using double sided adhesive tape and sputtered with gold before viewing under a JSM-5800 microscope (JEOL, Tokyo, Japan) (Mamvura et al., 2011).

2.3.6.2 Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) was also used for viewing of live and dead bacterial cells within the microparticles using a glycerol based staining method after staining with LIVE/DEAD® BacLight™ kit, according to Auty et al. (2001), with minor modifications. The stains, 1.5 µl of propidium iodide and 1.5 µl of SYTO9 were separately diluted into 1 ml of sterile water. Then, 100 µl of each dilute stain were added into 100 µl of

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glycerol and vortexed. Ten microlitres of this solution, was poured onto 10 µg of each freeze dried microparticles on a microscope slide. The slide was incubated in the dark for 15 min and viewed using a Zeiss 510 META confocal laser scanning microscope (Jena, Germany). Imaging was performed under the 100 X magnification lens and confocal illumination was provided by a 488 nm Ar/Kr laser.

2.3.7 Statistical analysis

Mean values and standard deviations were calculated from the data obtained from three independent trials. Data was analysed using one way ANOVA (analysis of variance) and a p-value less than 0.05 was considered to be statistically significant. Analysis was performed using JMP Statistical discovery software.

2.4 Results and Discussion

2.4.1 Comparison of formulations

A total of ten different formulations (section 2.3.3) encapsulating *Bifidobacterium longum* LMG 13197 in varying concentrations of inulin in Vegetal were produced. This was done to determine which concentration of inulin resulted in better protection and survival of *B. longum* during the freeze drying process. Two different methods for freeze drying were also tested to determine which one led to less reduction in viable cells. The bifidobacteria counts obtained from all formulations containing 1% and 1.5% (w/v) inulin concentrations were lower than 6 log cfu/g (Fig 2.1) and thus were not considered for further experiments. Since viable counts less than 6 log cfu/g indicate that the formulations did not protect the bacteria during the freeze drying process. Thus, the viability and functionality of the probiotic cells were greatly reduced and will not provide the required health benefits. The highest probiotic counts were obtained for 2%, 3.5% and 5% (w/v) inulin without centrifugation. These counts were 6.3, 6 and 6.2 log cfu/g respectively, and were not significantly different ($p>0.05$) (Fig 2.1). The losses in viable cells from an initial of 9.5 log cfu/g were 3.1, 3.5 and 3.2 log cfu/g for inulin (2%, 3.5% and 5% w/v) respectively. Encapsulation efficiencies of 82%, 80% and 81.4% were achieved for microparticles containing 2%, 3.5% and 5% (w/v) inulin respectively (Fig 2.2). These results indicate that the microparticles obtained with 2% (w/v)

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inulin performed slightly better than the others because they resulted in better protection of *B. longum* LMG 13197 during freeze drying process. Addition of more inulin in the formulation did not result in enhanced protection during freeze drying. Researchers elsewhere have also reported that addition of 2% (w/v) inulin enhanced the growth of probiotic cells in food products (Boeni and Pourahmad, 2012). Microparticles produced with 2% (w/v) inulin were therefore further characterized in this study.

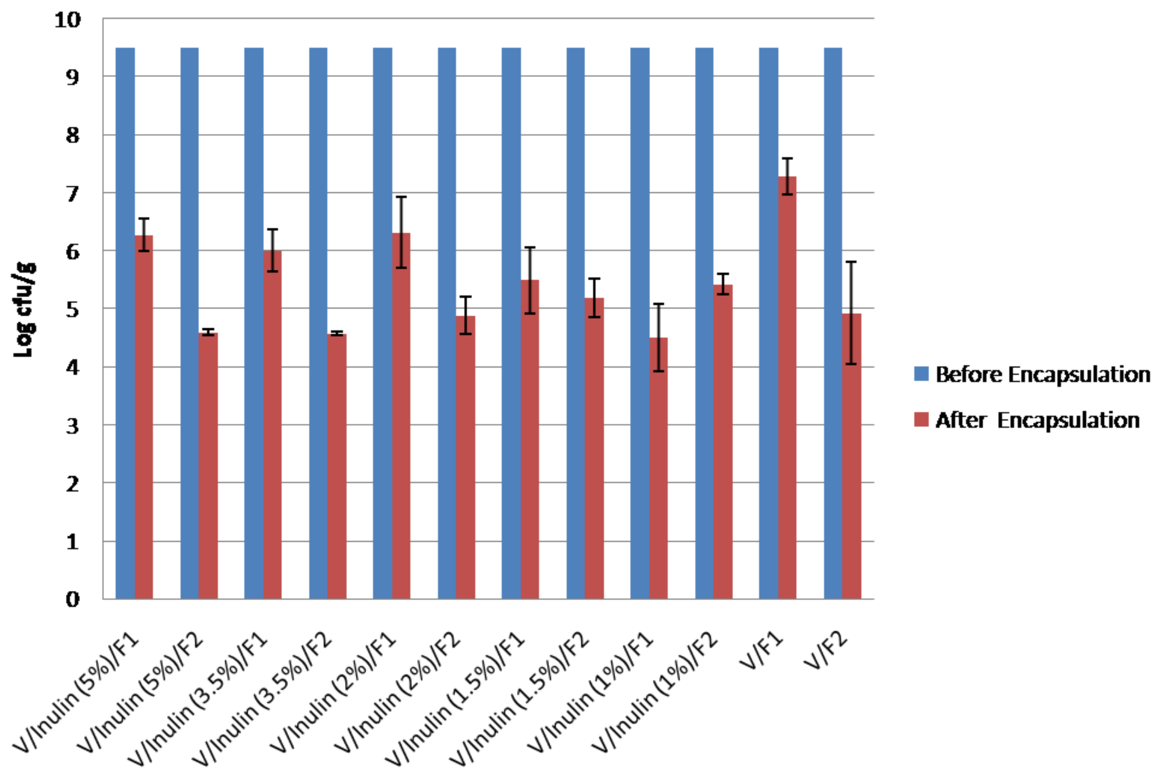

Figure 2.1 Viable numbers of bacteria released from the different microparticles before and after freeze drying in different formulations. F1= no centrifugation, F2= centrifugation + water, V= Vegetal BM 297 ATO. Each bar represents the mean of triplicate data from three independent trials; error bars represent standard deviations.

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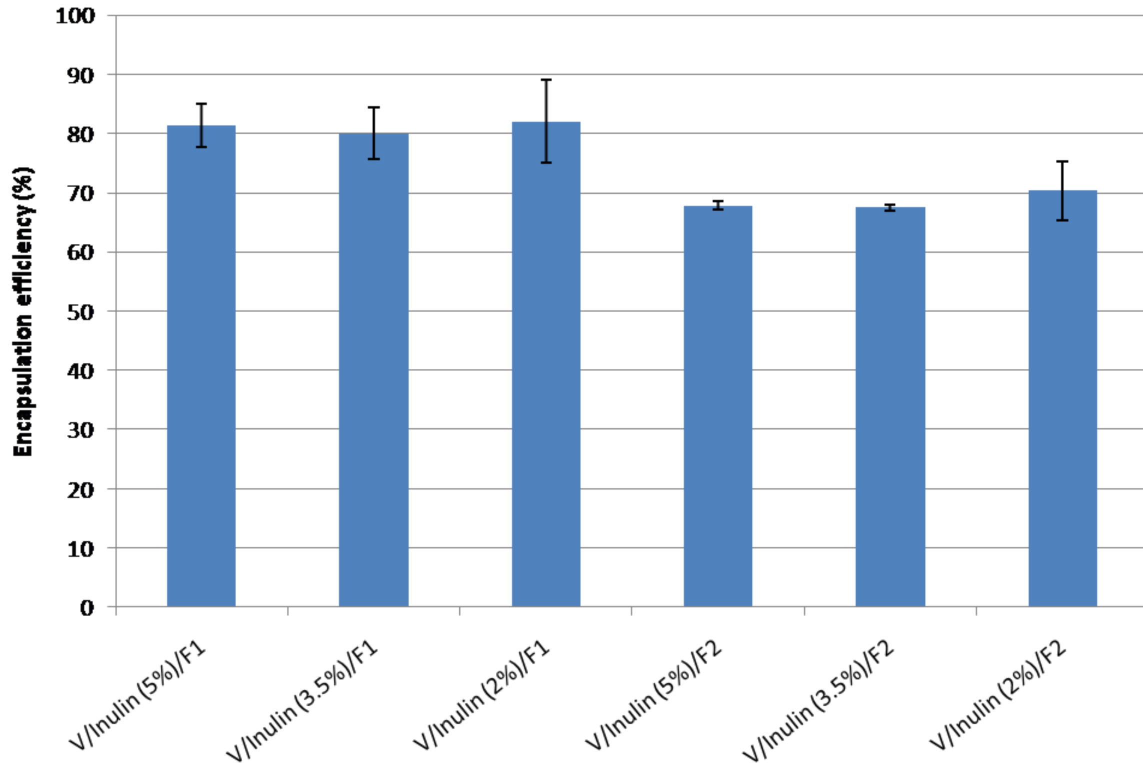

Figure 2.2 Encapsulation efficiencies of microparticles produced with formulations containing different inulin concentrations. F1= no centrifugation, F2= centrifugation + water, V = Vegetal BM 297 ATO. Each bar represents the mean of triplicate data from three independent trials; error bars represent standard deviations.

The bifidobacteria counts obtained from formulations without inulin were 7.3 and 4.9 log cfu/g without and with centrifugation step; respectively (Fig 2.1). The formulation with a count of 4.9 log cfu/g was not further analysed, for reasons previously stated. Therefore, our results show that high numbers of viable cells were obtained from non-centrifuged formulation (F1) without inulin and formulation (F1) with 2% (w/v) inulin (Fig 2.1). This shows that encapsulation with Vegetal-inulin matrix protected the cells during encapsulation process as a high number of them were realised after encapsulation. It was also observed that the formulations produced with centrifugation resulted in lower viable counts (Fig 2.1). Low bacterial counts could have been caused by the disruption of emulsion and release of cells during centrifugation. Gilbert et al. (1991) reported reduced viability in log phase bacterial cells after centrifugation at 5000 to 20 000 g for 5 to 20 min. Centrifugation results in bacterial compaction which causes bacterial cells to collide with one another, which in turn increases shear forces that damage their DNA and cell surface properties (Allan and Pearce,

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1979; Gilbert et al., 1991; Pembrey et al., 1999). Thus, bacterial cells suffer sub-lethal injuries which alter their metabolic and physiological abilities, such as salt or acidic tolerance, adhesion to surfaces, survival in selective media as well as their ability to form colonies (Wayber et al., 1994; Peterson et al., 2012).

2.4.2 Particle size distribution and encapsulation efficiency

After particle size analysis, the average particle size distribution of the microparticles produced is shown in Fig 2.3. The mean diameter obtained for microparticles produced with and without inulin were 33.4 ± 3.0 µm and 80.9 ± 20.3 µm (mean ± standard deviation, n= 6) respectively (Table 2.1). The D values (Table 2.1) indicate the percentage of the volume or weight of the particles that is smaller than the actual size. Therefore, D_{90} values of both formulations produced with and without inulin indicate that 90% of the particles had diameters less than 33.4 µm and 80.9 µm respectively and they are both within the required range (<100 µm) for microparticles for food applications (Hansen et al., 2002). However, results show that the addition of inulin resulted in the production of much smaller particle size (Table 2.1). This is desirable as smaller microparticles are stable, easier to handle and may not negatively affect the organoleptic properties of foods (Doleyres and Lacroix, 2005). These results are in agreement with the findings of Fritzen-Freire et al. (2012) who reported that addition of inulin resulted in a decrease in microparticle size, when compared to the control. However, Okuro et al. (2013) reported that the presence of inulin did not affect the lipid microparticle sizes, when compared to the control. Comparing the size of microparticles from other studies (Cui et al., 2006; Pop et al., 2012; Heidebach et al., 2012) with the lipid based microparticles produced in this study; it can be concluded that the presence of inulin in the formulation enhanced the food applications attributes of microparticles made from Vegetal BM 297 ATO.

High encapsulation efficiency indicates that a high number of viable cells were encapsulated and thus can administer the minimum amount required for health benefits (Champagne et al., 2005). Vegetal BM 297 ATO microparticles produced with and without inulin (without centrifugation step) gave encapsulation efficiencies of 82% and 88% respectively (Table 2.1). These results indicate that a high percentage of bacteria cells were retained in the matrix during encapsulation. Studies have reported that microcapsules with encapsulation

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efficiencies of 70% using an emulsion process, and have been shown to release high number of cells where needed (Heidebach et al., 2012). Pop et al. (2012) also reported an encapsulation efficiency of 66.87% for the encapsulation of *B. lactis* 300B with alginate/pullulan. When comparing the encapsulation efficiencies obtained in the aforementioned studies to those obtained in this study, it can be observed that although the presence of inulin reduced encapsulation efficiency, the use of Vegetal BM 297 ATO as a coating material for *B. longum* LMG 13197 resulted in higher encapsulation efficiencies.

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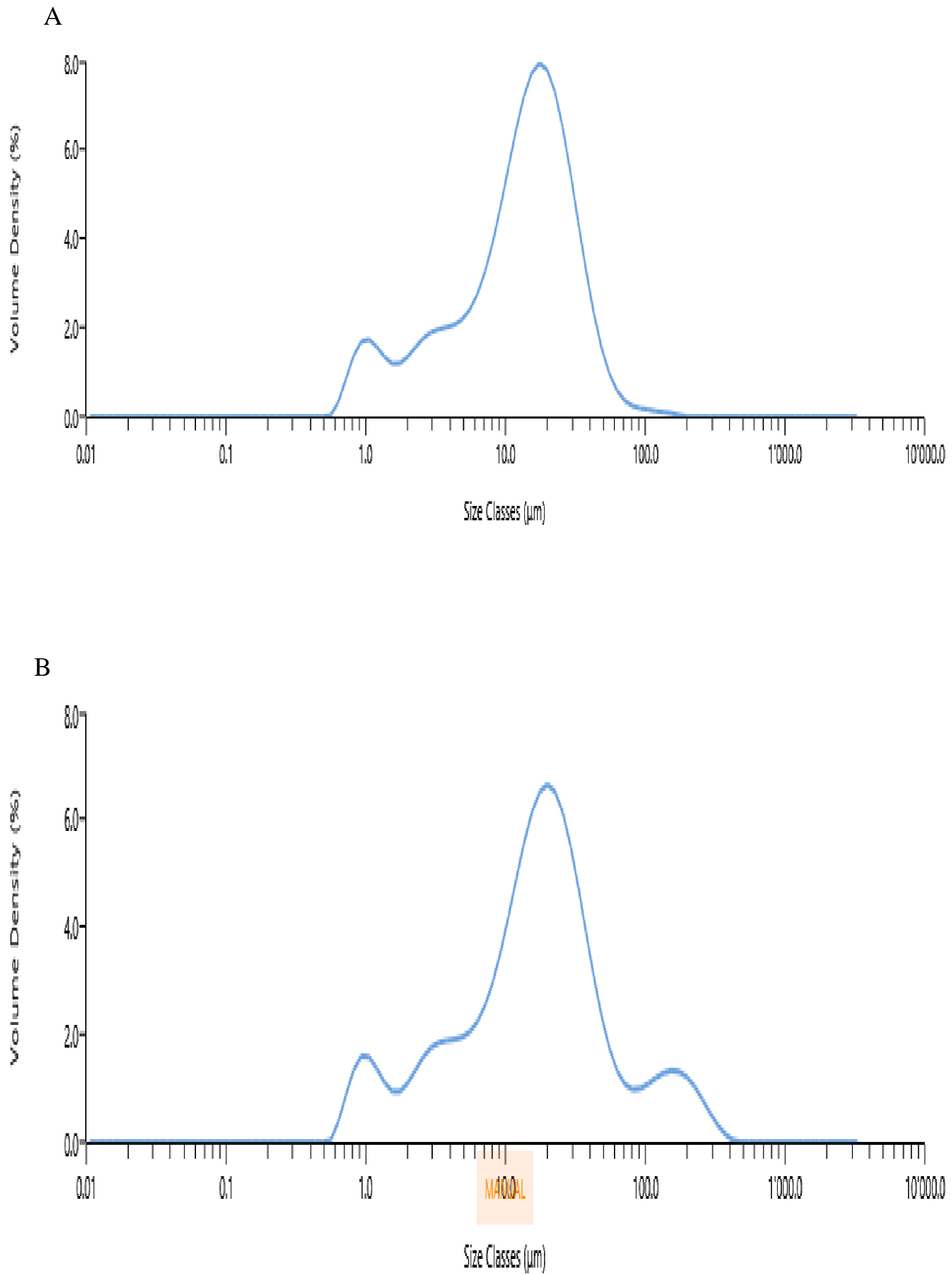

Figure 2.3 Average particle size distributions of Vegetal microparticles (A) with 2% inulin and (B) without inulin.

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Table 1 – Mean particles size and encapsulation efficiency of non-centrifuged Vegetal BM 297 ATO-inulin microparticles.

Microparticle formulation	**Mean particle size (µm ± standard deviation)**			**EE (%)**
	D10	D50	D90	
Without inulin	2.2 ± 0.1	17.1 ± 1.1	80.9 ± 20.3	88
With inulin	2.0 ± 0.1	13.8 ± 0.8	33.4 ± 2.9	82

Reported values are means of triplicate readings from three independent trials, EE = Encapsulation efficiency

2.4.3 Morphological characterization and bacterial loading within microparticles

Scanning electron microscopy showed a high loading of rod to coccoid shaped bacterial cells within the microparticles (Fig 2.4B and D) and free bacterial cells with no coverings around them for the unencapsulated sample (Fig 2.4E). The presence of coccoid cells within the microparticles is attributed to the change in bifidobacteria morphology during their exposure to the stressful cold and dry conditions associated with freeze drying process. Studies have shown that when bifidobacteria are exposed to environmental stresses, their rod shape appearance can change (Biavati et al., 2000; Boylston et al., 2004). There were no visible bacterial cells between or on the surfaces of the microparticles of both formulations (Fig 2.4A and C), which confirms that there was efficient encapsulation. Furthermore, SEM revealed irregular shaped microparticles, which exhibited rough surfaces with visible concavities and few pores (Fig 2.4A and C), as has been reported in other studies (Sheu and Marshall, 1993; Sultana et al., 2000; De Castro-Cislaghi et al., 2012; Fritzen-Freire et al., 2012). In this study, the presence of irregular shaped microparticles exhibiting pores and concavities can be attributed to the shrinkage of particles which occurs during drying processes. This process can change the physical, chemical and biological properties of the encapsulation matrix, which possibly reduces its ability to protect cells from stressful conditions (Fritzen-Freire et al., 2012; Martin-Dejardin et al., 2013). Porous microparticles are not desirable because

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presence of pores will result in premature release of bacteria from the microparticles (Ravi et al., 2008). However, since only few pores were observed, there is a possibility that the release of bacterial cells from the matrix will be minimal, though further investigation is required to prove this.

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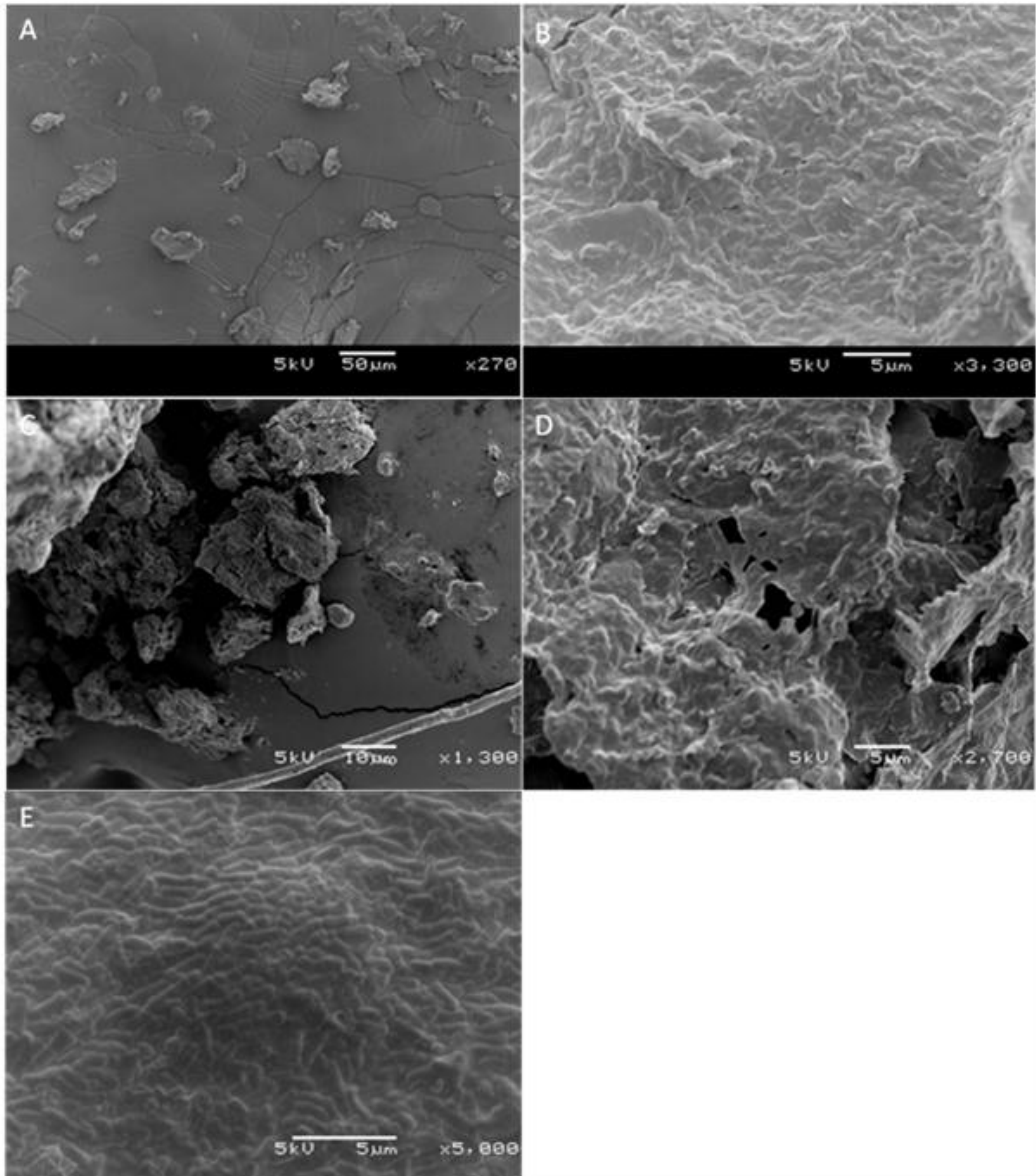

Figure 2.4 SEM images showing Vegetal BM 297 ATO microparticles with and without inulin (A and C) morphology and particle size, (B and D) distribution of *B. longum* LMG 13197 within the microparticles, (E) unencapsulated *B. longum* LMG 13197.

The confocal laser scanning microscopy images distinguish between dead and live bacterial cells. This is made possible by the use of a LIVE/DEAD BacLight kit, which has been utilised by various researchers (Alakomi et al., 2005; Berney et al., 2007). The kit contains

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two nucleic acid stains; SYTO9 (green fluorescence) which stains both live and dead cells and Propidium iodide (red fluorescence) which stains cells with compromised or damaged membranes (Alakomi et al., 2005). However, when both stains are taken up, the propidium iodide (PI) displaces the SYTO9 stains in damaged and dead cells (Alakomi et al., 2005). Auty et al. (2001) developed a glycerol based method, which was used in this study to allow for viewing of probiotic cells with minimal or no dissolution of the encapsulating matrix. The CSLM images showed that microparticles produced with and without inulin contained a high loading of fluorescent bifidobacteria cells (Fig 2.5A and B). Some of the cells were released and dispersed throughout and on the surface of the microparticles (Fig 2.5A and B), possibly increasing their exposure to detrimental factors such as oxygen dissemination into the matrix. The few cells that are released and dispersed throughout and on the surface of the microparticles suggests cells that were not completely embedded deep within the matrix, and are thus close to the surface of the matrix. The partially dissolved matrix fluoresced green at a high intensity than some of the bacterial cells (Fig 2.5A and B). There was a mixture of live (green) and compromised (yellow) cells in microparticles produced with inulin (Fig 2.5A). The yellow colour indicates that the cell membrane had an intermediate degree of damage, which allowed PI to penetrate (Liu et al., 2007). Studies have reported that the presence of live and compromised cells in freeze dried bifidobacteria is to be expected; however, the compromised cells may recover and become viable under suitable conditions (Pop et al., 2012). There was a mixture of live (green) and dead (red) cells in microparticles produced without inulin (Fig 2.5B), with majority of the visible cells stained red. The presence of red cells indicates that the cell membranes possibly suffered irreversible cell damage during freeze drying, thus allowed PI to penetrate (Alakomi et al., 2005; Liu et al., 2007). Therefore, our results show that the addition of inulin enhanced protection of *B. longum* LMG 13197 as there were fewer dead (red) cells for bacteria encapsulated in a formulation containing inulin.

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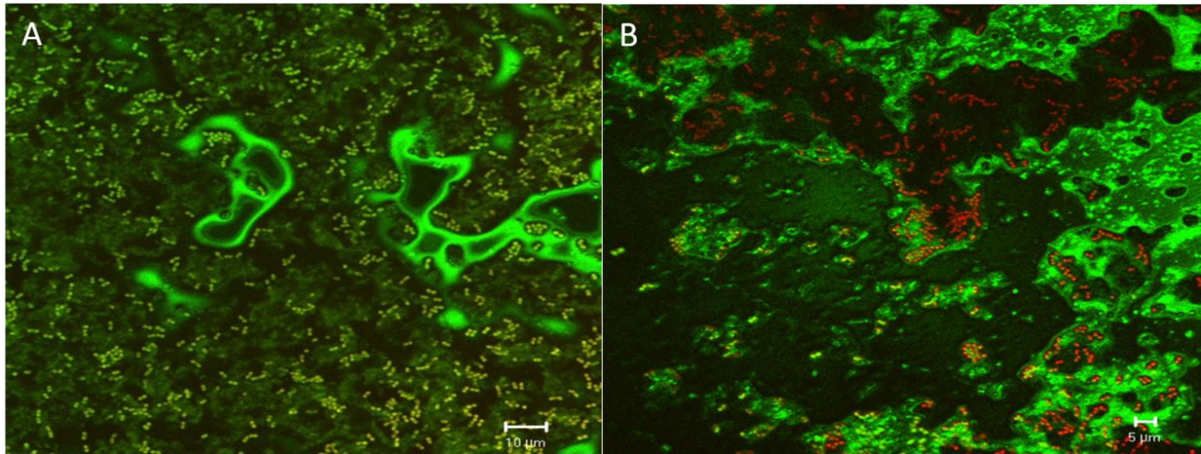

Figure 2.5 CLSM images of stained *B. longum* LMG 13197 cells within Vegetal BM 297 ATO microparticles, (A) with inulin, (B) without inulin. Live cells (green), damaged cells (yellow) and dead cells (red).

2.5 Conclusion

This study shows the successful production of lipid-based microparticles using Vegetal for the encapsulation of *Bifidobacterium longum* LMG 13197 by freeze drying method. Addition of inulin reduced the size (33.4 µm) but did affect the morphology of microparticles, with an associated slight reduction of the encapsulation efficiency. Nevertheless, the encapsulation efficiency was still high enough to indicate that a large number of cells were retained in the matrix. Thus, the results show that synbiotic Vegetal-inulin microparticles produced by freeze drying have the potential for use as a functional food additive, as they are likely to deliver sufficient numbers of viable bacteria.

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CHAPTER 3

Survival of Vegetal-inulin encapsulated *Bifidobacterium longum* LMG 13197 during storage and simulated gastrointestinal fluids transit

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3.1 Abstract

Probiotics are sensitive to certain environmental factors which include gastric acid, temperature and oxygen. Over time the numbers of viable bacteria decrease to numbers below the recommended quantity for beneficial effects to be observed. Microencapsulation can be used to protect the cells during gastrointestinal transit and extend their shelf life, increasing their suitability for use as a health-promoting food additive. This study investigated the survival of Vegetal-inulin BM 297 ATO encapsulated *Bifidobacterium longum* LMG 13197 when exposed to simulated gastrointestinal fluids and different storage conditions. Samples of Vegetal BM 297 ATO encapsulated bifidobacteria (with and without inulin) and unencapsulated bacteria as control were exposed to simulated gastric fluid (SGF) (pH 2.0) for 2 h, and subsequently to simulated intestinal fluid (SIF) (pH 6.8) for 6 h. Samples taken at various times were analysed using the fluorochrome microplate assay and plate counting methods using MRS-agar supplemented with 0.05% cysteine hydrochloride. Vegetal BM 297 ATO encapsulated bifidobacteria (with and without inulin) and unencapsulated bifidobacteria were also were also stored in glass bottles at 4ºC and 25ºC for 6 weeks. Numbers of viable cells were determined on weekly basis using plate counting, fluorochrome microplate assay and flow cytometry. Vegetal matrix displayed little release of cells in SGF and significant release in SIF. The addition of inulin at 2% (w/v) improved the protection efficiency of Vegetal, and thus provided better protection to the cells during exposure to simulated gastrointestinal fluids. Compared to the unencapsulated cells, Vegetal encapsulation (with and without inulin) improved the survival of *B. longum* LMG 13197 during 6 weeks of storage at 25ºC. Encapsulation showed no significant protection at 4ºC; but Vegetal-inulin matrix did extended shelf life to 5 weeks at 4ºC. Therefore, Vegetal-inulin encapsulated bacteria can be applied in food industries with favourable storage conditions at refrigeration temperatures.

Keywords: Probiotics, Inulin, Simulated Gastric fluid, Simulated Intestinal Fluid, Vegetal BM 297 ATO, *Bifidobacterium longum* LMG 13197, Microencapsulation.

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3.2 Introduction

Probiotics are currently used as dietary supplements or incorporated into food products (Vasiljevic and Shah, 2008; Kramer et al., 2009). In order for them to exert beneficial effects, they must remain viable and in sufficient amounts throughout their shelf life period and upon arrival in the colon (Bruno and Shah, 2003; Guarner and Malagelada, 2003; Song et al., 2012). The minimum amount of probiotics required to exert beneficial effects ranges from 10^6 to10^8 cfu/ml (Sanders and Veld, 1999). Studies have reported that the number of viable probiotic cultures decline before consumption, thus resulting in failure of products to supply the minimum amount required for health benefits (Shah et al., 2000; Vinderola et al., 2000; Suita-Cruce and Goulet, 2001). Factors which affect the viability of probiotic cultures during storage and gastrointestinal transit have been reported (Lian et al., 2002; Piano et al., 2006), and they include among others, temperature, oxygen, moisture and the presence of acid and bile in the stomach and small intestine, respectively (Thantsha et al., 2009; Weinbreck et al., 2010; Brinques et al., 2011). Bifidobacteria, one of the common probiotic species, are sensitive to these stressful factors and do not survive transit through the gastrointestinal tract (GIT), nor remain viable in food products during storage (Adhikari et al., 2000; Lourens-Hattingh and Viljoen, 2001; Hansen et al., 2002).

Microencapsulation is an effective technique used to protect and improve viability of probiotic cultures during storage and GIT transit (Sultana et al., 2000; Hansen et al., 2002; Weinbreck et al., 2010). One of the microencapsulation techniques, known as freeze drying or lyophilisation, has been purported to improve bacterial resistance to gastrointestinal fluids and promote extended shelf life of probiotics during storage (Heidebach et al., 2010; Okuro et al., 2013). Freeze drying does however; affect the physiological state of bacteria resulting in loss of viable cells (Rault et al., 2007). There is therefore an opportunity to develop new techniques of encapsulation to minimise cell death during the process. An approach which involves the addition of prebiotics before freeze drying for the improved survival of probiotics has been explored (Özer, et al., 2005; Akalin and Erisir, 2008; Heydari et al., 2011). It is worth mentioning that prebiotics are not hydrolysed in the upper part of the GIT, and therefore have the potential to protect probiotics throughout their journey to the colon (Reyed, 2007).Varying results from freeze dried combinations of probiotics and prebiotics have been reported, depending on the encapsulating matrix used (Capela et al., 2006; Pop et

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al., 2012; Cheng et al., 2013; Dianawati et al., 2013). To date however, there has been limited research on the use of lipid based matrixes in combination with prebiotics for protection of bifidobacteria during storage and GIT transit. This study aimed to assess the effect of encapsulation in Vegetal BM 297 ATO in conjunction with inulin on the survival of *Bifidobacterium longum* LMG 13197 during storage and in simulated gastrointestinal fluids.

3.3 Materials and Methods

3.3.1 Reagents and bacterial cultures

Biogapress Vegetal BM 297 ATO was obtained in powdered form from (Gattefossé SAS). *Bifidobacterium longum* LMG 13197 cultures were obtained from BCCM/LMG Culture collection (Belgium) as 20% glycerol stocks in MRS broth at -70°C and revived according to the manufacturer's specifications. Inulin (purity: 95%) (purity: 99%), polyvinyl alcohol (PVA) 87-89% partially hydrolysed (Mw: 13000-23000 Da), lactose monohydrate were obtained from Sigma Aldrich, South Africa, while dichloromethane (DCM) (analytical grade, purity: 99%) was obtained from Sigma Aldrich Laborchemikalien, Seelze.

3.3.2 Bacterial culturing and enumeration

Frozen stocks of *B. longum* LMG 13197 were allowed to thaw and streaked out onto MRS-cys-HCl agar plates and incubated in anaerobic jars with Anaerocult A gaspaks and Anaerocult C strip for 48 h at 37°C to obtain single colonies. A single colony was then inoculated into 5 ml MRS-cys-HCl broth and grown anaerobically overnight at 37°C. One millilitre of this overnight culture was then subcultured twice on separate occasions into 100 ml of MRS-cys-HCl broth and incubated anaerobically at 37°C overnight. One millilitre of the culture was used for bacteria enumeration after dilution in Ringer's solution. A subsample of 100 µl was taken from the broth culture and suspended in 900 µl of ¼ strength Ringer's solution. A 10-fold serial dilution up to 10^{-10} dilution was performed and 100 µl of each dilution was plated out onto MRS-cys-HCl agar in triplicates and plates were incubated (General purpose incubator, Shel Lab) at 37°C for 72 h under anaerobic conditions. After incubation, number of viable cells were determined and recorded as cfu/ml. After

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enumeration, 3 ml of the remaining culture was used for bacterial encapsulation while the rest was stored in 10% sterile glycerol at -20°C.

3.3.3 Bacteria encapsulation

Bacteria encapsulation was done using the method of Pimentel-Gonzalez et al. (2009) with modifications. The stored *B. longum* LMG 13197 cultures was allowed to thaw and 1 ml was subcultured into three 250 ml flasks containing 100 ml MRS-cys-HCl broth, and incubated anaerobically at 37°C for 48 h. After incubation, bacteria were harvested by centrifugation, using an Eppendorf centrifuge 5804R (cooled to 4°C) at 20 800 g for 15 min. The pelleted cells weighed 0.78 g (approximately 4.8×10^9 cfu/ml) and were washed once with Ringer's solution and kept at 4°C for 5 min before encapsulation. The first emulsion was prepared by suspending the bacterial pellet into 1 ml of 2% (w/v) inulin. The bacteria-inulin mixture was then added to 1 ml of 2% (w/v) poly-vinyl-alcohol (PVA). The resulting suspension was subsequently added to 10 ml dichloromethane (DCM) containing Vegetal BM 297 ATO at a final concentration of 10% (w/v). The resulting emulsion was homogenized at 8000 rpm for 5 min using a Silverson, L4R, NIMR homogenizer and left to stand at 25°C. The second emulsion was prepared by mixing 15 ml of PVA 2% (w/v) and 5 ml of lactose 5% (w/v). The first emulsion was mixed into the second emulsion and homogenised at 8000 rpm for 5 min using a Silverson, L4R, NIMR homogenizer. The stable emulsion was left to stand in the fume hood for 5 h for DCM evaporation. After evaporation of DCM, the sample was frozen at - 20°C overnight. This was followed by freeze drying using a Virtis bench top, SLC, freeze dryer for 3 days at -75°C. The freeze dryer was set at a condenser temperature and vacuum pressure of – 60°C and 0.26 millitor, respectively.

The same protocol was used to prepare Vegetal BM 297 ATO microparticles encapsulating *B. longum* LMG 13197 without inulin, except bacterial pellet was re-suspended in 1 ml of deionised water before mixing with 1 ml of 2% (w/v) poly-vinyl-alcohol (PVA). The control sample (unencapsulated cells) was prepared by resuspending B. *longum* LMG 13197 cells (approximately 4.8×10^9 cfu/ml) into 25 ml of sterile deionised water and fast-frozen in liquid nitrogen for 5 min. The fast-frozen cells were then frozen at – 70ºC for 1 h before freeze drying in a Virtis bench top, SLC, freeze dryer for 3 days at -75°C. After freeze drying, all

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the samples were stored in tightly sealed sterile Schott bottles at 4°C for 1 h until further analysis.

3.3.4 Determination of total bacteria encapsulated

Encapsulated bacteria (0.1 g) was suspended in 900 µl of DCM and vortexed for 30 s. Mixture was spun down using a MiniSpin, Eppendorf at 12 100 g for 30 s and the supernatant discarded. The pellet was then re-suspended in 900 µl of ¼ strength Ringer's solution. A tenfold serial dilution was performed and 100 µl of each dilution was plated out in triplicate onto MRS-cys-HCl agar using the pour plate method. The plates were incubated at 37°C for 72 h in anaerobic jars with Anaerocult A gaspaks and Anaerocult C test strip to indicate anaerobic conditions.

3.3.5 Preparation of simulated gastrointestinal fluids

SGF was prepared according to Lian et al. (2003). Briefly, pepsin (P7000, 1: 10000, ICN) (3 g/L) was suspended in sterile NaCl solution (0.5% w/v). The pH of the solution was adjusted to pH 2.0 with 12 M HCl, then filter sterilized through a 0.45 µm filter membrane (Millipore). SIF was prepared by dissolving 6.8 g of monobasic potassium phosphate (Sigma, St. Louis, MO, USA) in 250 ml of distilled water. This was followed by addition of 77 ml of 0.2 M NaOH and 500 ml of distilled water. The solution was vortexed for 30 min and then 10 g of pancreatin (P-1500, Sigma, St. Louis, MO, USA) was added and mixed. The solution was adjusted to pH 6.8 with 0.2 M NaOH or 0.2 M HCl. The total volume of the solution was made up to 1000 ml, followed by filter sterilization through a 0.45 µm filter membrane (Millipore). The simulated gastrointestinal fluids were freshly prepared for each experiment.

3.3.5.1 Survival of encapsulated bacteria in simulated gastric fluid (SGF)

One gram of unencapsulated and encapsulated bacteria samples were dispensed into separate test tubes containing 9 ml of SGF (pH 2.0). The tubes were vortexed for 30 s and incubated in a shaker incubator (Lasec, LM-575R) at 37°C for 2 h (Sun and Griffiths, 2000). One millilitre subsamples were withdrawn from the tubes at intervals 0, 30, 60 and 120 min from tubes containing the unencapsulated cells after vortexing and from tubes containing the

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encapsulated samples after gentle pipetting. Bacteria in the subsamples were then enumerated using viable plate count (VPC) and fluorochrome microplate assay (FMA) according to the method described by Alakomi et al. (2005).

3.3.5.2 Survival of encapsulated bacteria in simulated intestinal fluid (SIF)

After taking the 2 h subsamples for bacterial enumeration from SGF survival tests, the remaining suspensions of both the unencapsulated and encapsulated samples were pelleted by centrifugation using a Labnet Prism^{TM} Microcenrifuge at 7267 g for 5 min. The pellets were resuspended in 9 ml of SIF (pH 6.8). Tubes were incubated in a shaker incubator (Lasec, LM-575R) at 37°C for 6 h. One millilitre subsamples were taken at intervals 0, 2, 4 and 6 h (Picot and Lacroix, 2004) after mixing for bacterial enumeration using VPC and FMA.

3.3.5.3 Survival of encapsulated B. *longum* during storage

Duplicate samples of 1 g each for unencapsulated and encapsulated bacteria with or without inulin were weighed out and stored in glass vials at 4°C and 25°C for 6 weeks. Subsamples (0.1 g) were taken once a week and analysed for bacterial viability using flow cytometry assay (FCA), FMA and VPC.

3.3.6 Enumeration of cells

3.3.6.1 After exposure to simulated gastrointestinal fluids

The subsamples obtained after survival in SGF and SIF were serially diluted in ¼ strength Ringer's solution. Then 100 µl of each dilution was pour plated onto MRS agar supplemented with 0.05% cys-HCl in triplicates. Plates were incubated at 37°C for 72 h anaerobically in anaerobic jars with Anaerocult A gaspaks and Anaerocult C test strips.

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3.3.6.2 After storage

The encapsulated subsamples (0.1 g) obtained were suspended in 900 µl of DCM and vortexed for 30 s to release the bacterial cells from the encapsulating matrix. The mixture was spun down using a MiniSpin Eppendorf at 12 100 g for 30 s and the supernatant discarded. The pellet was then re-suspended in 900 µl of ¼ strength Ringer's solution. Unencapsulated subsamples (0.1 g) were suspended in 900 µl of ¼ strength Ringer's solution. A tenfold serial dilution was performed for all the samples and 100 µl of each dilution was plated out in triplicate onto MRS-cys-HCl agar plates using the pour plate method. The plates were incubated at 37°C for 72 h in anaerobic jars with Anaerocult A gaspaks and Anaerocult C test strip to indicate anaerobic conditions.

3.3.7 Fluorochrome microplate assay

3.3.7.1 Preparation of standard curve

The standard curve preparation was set up as follows: One hundred microlitres of *B. longum* LMG 13197 was inoculated into 25 ml of MRS broth supplemented with 0.05 % cysteine hydrochloride, and then incubated in a shaker incubator at 37°C in anaerobic jars with Anaerocult A gaspaks and Anaerocult C test strips for 72 h. Bacteria from this culture were harvested by centrifugation using the Eppendorf centrifuge 5804R (cooled to 4°C) at 20 800 g for 15 min. The pellet obtained was re-suspended in 2 ml of 0.85 % NaCl in a Falcon tube. One milliliter of the suspension was mixed with 20 ml of 0.85 % NaCl in two separate Falcon tubes labeled live and dead. Sample labeled dead was heat treated in a microwave for 2 min to kill the bacteria. Both live and dead bacteria samples were centrifuged using the Eppendorf centrifuge 5804R (cooled to 4°C) at 20 800 g for 15 min to pellet the cells. Pellets were re-suspended in 10 ml of 0.85 % NaCl. The cultures were adjusted to optical density at 600 nm (OD_{600}) of ~0.4 using a Spectronic® 20 Genesys™ spectrophotometer. Different volumes of live and dead bacteria (Table 3.1) were mixed to achieve different proportions of live: dead cells for determining a standard curve. Viable bacterial counts were determined for each bacterial mixture and used to correlate the green-to-red ratio obtained after staining. These values were used to plot the standard curve.

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Table 3.1: Proportions of live:dead cells used to calculate standard curve.

Ratio of live : dead cells	Live cell suspensions (ml)	Dead cell suspensions (ml)
0:100	0	2.0
10:90	0.2	1.8
50:50	1.0	1.0
90:10	1.8	0.2
100:0	2.0	0

3.3.7.2 Staining of bacterial suspensions and fluorochrome microplate assay

The staining of bacteria cultures was performed using a LIVE/DEAD ® Baclight ™ bacterial viability kit. The bacterial viability kit utilizes PI (red fluorescence at 620 nm) and SYTO9 (green fluorescence at 530 nm). The staining solution was prepared according to the manufacturer's instructions to obtain 3.34 mM of SYTO9 and 20 mM of PI stock solutions dissolved in dimethyl sulfoxide. From these concentrations, a 2x staining solution was made by mixing 6 µl of PI and 6 µl of SYTO9 in 2.0 ml of filter sterilized water. Bacteria suspensions were then stained by mixing 100 µl of the bacterial suspension (approximately 1×10^9 cfu/ml) with 100 µl of the 2x staining solution in triplicates into a black 96 well fluorescence microtitre plate, as previously described (Alakomi et al., 2005). The microtitre plates were covered with foil and incubated in the dark for 15 min at room temperature. Fluorescence of bacterial suspensions was measured using the automated Fluorometer Fluoroskan Ascent FL (lab systems, Helsinki, Finland). The excitation and emission of red fluorescence were 530 nm and 635 nm respectively, while those of green fluorescence were 485 nm and 538 nm respectively. The intensities of the green-to-red ratio was used to compute the colony forming unit in each sample based on the standard curve previously prepared for *B. longum* LMG 13197 (Fig 3.1).

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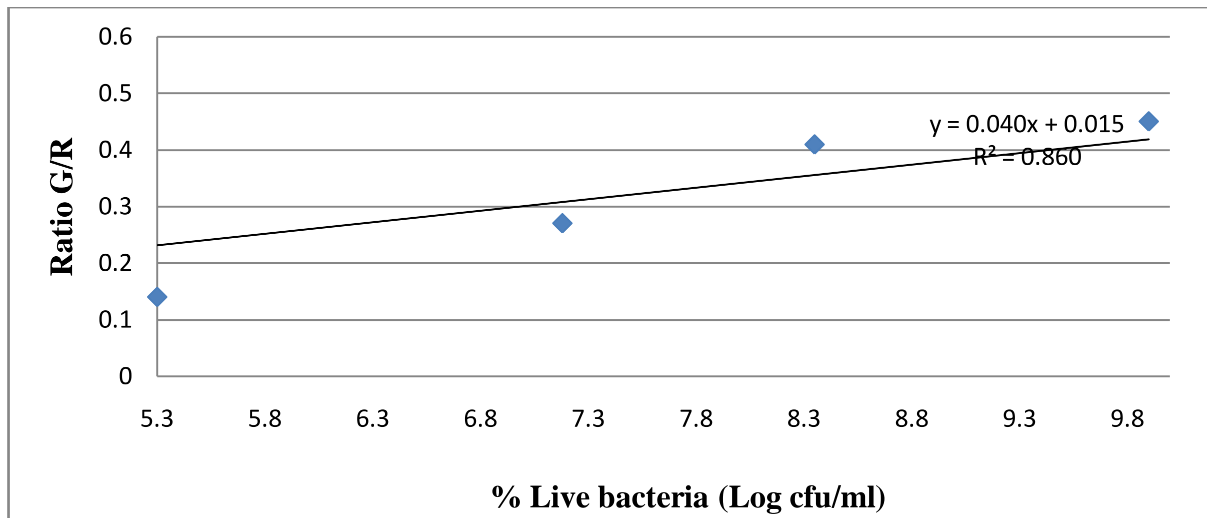

Fig 3.1 Standard curve for *B. longum* LMG 13197 showing relationship between proportion of live bacteria (log cfu/ml) and green/red fluorescence ratio (Ratio $_{G/R}$).

3.3.7.3 Fluorochrome microplate assay of B. *longum* LMG 13197 cells after exposure to simulated gastrointestinal fluids and storage

One millilitre bacterial samples obtained after survival in SGF and SIF were transferred to Eppendorf tubes and centrifuged at 12 100 g for 2 min. The pellets were then re-suspended in 0.85 % NaCl (Alakomi et al., 2005). The bacterial suspensions were adjusted to optical density at 600 nm (OD_{600}) of ~0.4 using a Spectronic® 20 Genesys™ spectrophotometer. Staining was performed by mixing 100 µl of the bacterial suspension with 100 µl of the 2x staining solution (6 µl of PI and 6 µl of SYTO9 in 2.0 ml of filter sterilized water) in triplicates into a black 96 well fluorescence microtitre plate. The microtitre plates were covered with foil and incubated in the dark for 15 min at room temperature. Fluorescence of bacterial suspensions was measured using the automated Fluorometer Fluoroskan Ascent FL (lab systems, Helsinki, Finland) as previously described.

The subsamples (0.1 g) obtained after survival during storage for unencapsulated cells were suspended in 900 µl of ¼ Ringer's solution and centrifuged at 12 100 g for 5 min to obtained pellets. The subsamples (0.1 g) obtained from the encapsulated cells were suspended in 900 µl of DCM and vortexed for 30 s to release the bacterial cells from the lipid matrix. This suspension was centrifuged at 12 100 g for 5 min and pellets were washed with ¼ Ringer's

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solution by further centrifugation at 12 100 g for 5 min. The pellets obtained from both unencapsulated and encapsulated samples were then re-suspended in 0.85 % NaCl. The bacterial suspensions were adjusted to optical density at 600 nm (OD_{600}) of ~0.2 using a Spectronic® 20 Genesys™ spectrophotometer. Staining was performed by mixing 100 µl of the bacterial suspension with 100 µl of the 2x staining solution (6 µl of PI and 6 µl of SYTO9 in 2.0 ml of filter sterilized water) in triplicates into a black 96 well fluorescence microtitre plate. The microtitre plates were covered with foil and incubated in the dark for 15 min at room temperature. Fluorescence of bacterial suspensions was measured using the automated Fluorometer Fluoroskan Ascent FL (lab systems, Helsinki, Finland) as previously described.

3.3.8 Flow cytometry assay

3.3.8.1 Preparation of standard curve

One hundred microlitres of *B. longum* LMG 13197 was grown in 25 ml of MRS broth supplemented with 0.05 % cysteine hydrochloride and incubated in a shaker incubator at 37°C under anaerobic conditions for 72 h. Twenty five milliliter of this culture was harvested by centrifugation at 20 800 g for 15 min. The pellet obtained was re-suspended in 2 ml of phosphate buffered saline (PBS) in a Falcon tube. One milliliter of the suspension was mixed with 20 ml of PBS in two separate Falcon tubes labeled live and dead. Sample labeled dead was heat treated in a microwave for 2 min to kill the bacteria. Both live and dead samples were centrifuged at 20 800 g for 15 min to pellet the bacterial cells. The resulting pellets were re-suspended in 10 ml of PBS and the OD_{600} adjusted to ~0.4 using a spectrophotometer (Spectronic® 20 Genesys™). Different volumes of live and dead bacteria were mixed and used to determine the standard curve as indicated in Table 3.1. Viable counts was determined for each bacterial suspension and used to correlate the percentage of live bacteria obtained from flow cytometry to plot the standard curve (Fig 3.2).

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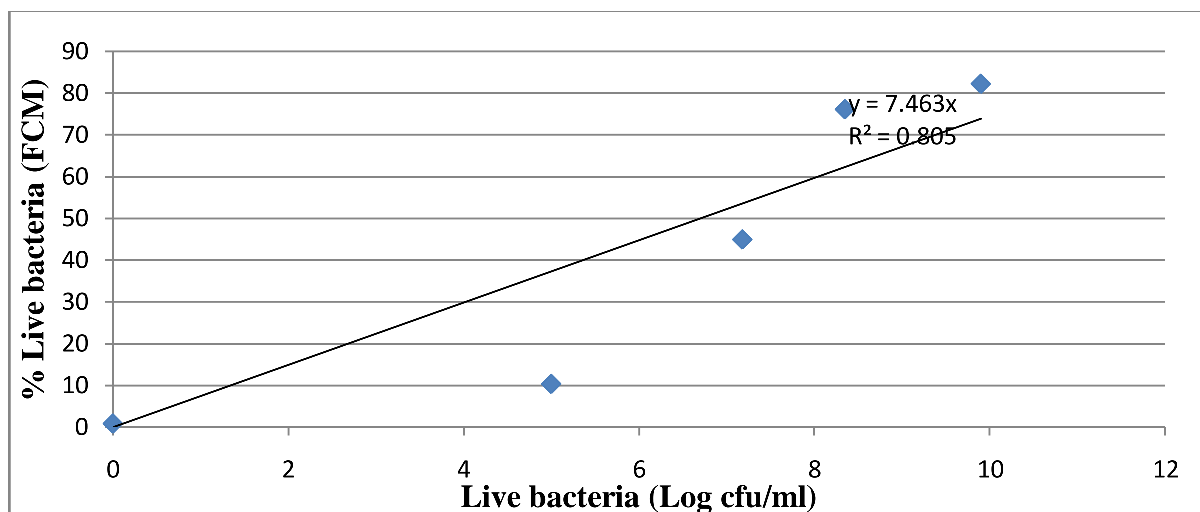

Fig 3.2 Standard curve for *B. longum* LMG 13197 showing relationship between proportion of live bacteria (log cfu/ml) and measured FCM live cells.

3.3.8.2 Staining of bacterial suspensions and flow cytometry assay

Staining of bacteria cultures was performed in triplicates using Live/Dead BacLight bacterial viability kit L7012 (Molecular Probes). Hundred microlitres of the bacterial suspension (approximately 10^9 cfu/ml) was mixed with 100 µl of staining mix (1.5 µl of SYT09, 1.5 µl of PI and 1 ml of filter sterilized water) into eppendorf tubes. The tubes were covered with foil and incubated in a dark cupboard for 15 min. After incubation, the samples were transferred to a glass cuvette for analysis using a flow cytometer (FAC-Scan flow cytometer, Becton-Dickinson, Le Pont de Claix, France). The FAC-Scan analyzes cells passing through the sheath fluid using five band filters which include forward angle light scatter (FSC), side angle light scatter (SSC) and 3 fluorescence signals. These signals recognise fluorescent colours at different wavelengths and they include 530 nm {collects green fluorescence of SYT09, 30 nm band width, band pass filters ranging from 675- 715 nm (FITC)}, 585 nm {collects yellow-orange fluorescence} and 695 nm {collects red fluorescence of PI, 40nm band width, band pass filters ranging from 515-545nm (Per-C-P)}. FACSDIVA Version 6.1.3 was used to perform data analysis and the spectral overlap between the fluorescences emitted by both stains was removed by adjusting the compensation values and voltage threshold.

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3.3.8.3 Preparation of control samples

One hundred microliters of *B. longum* LMG 13197 was grown in two flasks containing 25 ml of MRS broth supplemented with 0.05 % cysteine hydrochloride and incubated in a shaker incubator at 37°C under anaerobic conditions for 72 h. After incubation, the flasks containing bacteria cultures were labelled live and dead. Sample labeled dead was heat treated in a microwave for 2 min to kill the bacteria. One millilitre subsample of the live sample was transferred into two Eppendorf tubes and labelled live and unstained respectively, while 1 ml subsample of the dead sample was transferred into an Eppendorf tube and labelled dead. These samples represented the controls used to differentiate the various bacterial populations into quadrants and to calculate the compensation of spectral overlap of different fluorochromes. The eppendorf tubes containing live, dead and unstained samples were centrifuged at 20 800 g for 15 min to pellet the bacterial cells. The resulting pellets were re-suspended in 1 ml of PBS and the OD_{600} adjusted to ~0.4 using a spectrophotometer (Spectronic® 20 Genesys™). The staining of bacteria cultures was performed in triplicates using Live/Dead BacLight bacterial viability kit L7012 (Molecular Probes). The Eppendorf tubes containing live and dead bacterial cells were stained separately with 1.5 µl of SYT09, 1.5 µl of PI and 100 µl of mixed stains (1.5 µl of SYT09, 1.5 µl of PI and 1 ml of filter sterilized water). After staining the cells, the tubes were covered with foil and kept in a dark cupboard for 15 min before performing flow cytometry analysis as described in section 3.3.8.2.

3.3.8.4 Flow cytometry assay of cells after storage

The subsamples (0.1 g) obtained after storage for unencapsulated cells were suspended in 900 µl of ¼ Ringer's solution and cells were pelleted by centrifugation at 12 100 g for 5 min. Subsamples (0.1 g) of encapsulated cells were suspended in 900 µl of DCM and vortexed for 30 s to release the bacterial cells from the encapsulating matrix. This suspension was centrifuged at 12 100 g for 5 min and pellets were washed with ¼ Ringer's solution by further centrifugation at 12 100 g for 5 min. Pellets obtained from unencapsulated and encapsulated samples were then re-suspended in 1 ml of PBS. The bacterial suspensions were adjusted to optical density at 600 nm (OD_{600}) of ~0.4 using a Spectronic® 20 Genesys™ spectrophotometer. Dual staining was performed in triplicates with Live/Dead BacLight

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bacterial viability kit L7012 (Molecular Probes), where the bacterial suspensions were stained with 100 µl of (1.5 µl of SYT09, 1.5 µl of PI and 1 ml of filter sterilized water). After staining the cells, the tubes were covered with foil and incubated in a dark cupboard for 15 min before flow cytometry analysis as previously described.

3.3.9 Statistical analysis

Mean values and standard deviations were calculated from the data obtained from three independent trials. Data was analysed using one way ANOVA (analysis of variance) and a p-value less than 0.05 was considered to be statistically significant. Analysis was performed using JMP Statistical discovery software.

3.4 Results and Discussions

3.4.1 Survival of encapsulated bacteria in simulated gastrointestinal fluids

Unencapsulated and encapsulated bacteria were exposed to simulated gastric fluids (pH 2) for 2 hours and subsequently to intestinal fluids (pH 6.8) for 6 hours. The results obtained are shown in Figure 3.3. During exposure of unencapsulated bacteria to SGF, results showed a continuous decrease in the number of viable cells from an initial count of 8.60 $\log_{10}$ cfu/ml to a final count of 6.26 $\log_{10}$ cfu/ml (2.34 log decrease) and from 8.92 to 7.50 $\log_{10}$ cfu/ml (1.42 log decrease) after 2 h for VPC and FMA respectively (Fig 3.3). Similar results have been reported following exposure of unencapsulated bacterial cells to SGF by researchers elsewhere. Hansen et al. (2002), showed a 3 to 4 $\log_{10}$ cfu/ml decrease in *B. longum* Bb-46 after 2 h while De Castro-Cislaghi et al. (2012) reported a 1.51 log decrease in unencapsulated *B. lactis* Bb-12 at pH 2.

The exposure of Vegetal encapsulated bacteria to SGF led to an initial decrease of viable counts from 7.16 to 6.95 $\log_{10}$ cfu/ml and from 7.30 to 7.10 $\log_{10}$ cfu/ml in the first 30 min as indicated by VPC and FMA respectively (Fig 3.3). This initial drop may be due to the immediate release of cells that were not enclosed deep within the matrix. This was followed by an increase in the number of cells to 7.30 and 7.88 $\log_{10}$ cfu/ml for VPC and FMA respectively after 2 h (Fig 3.3). This suggests that the matrix disintegrated in the low pH and

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released some of the encapsulated cells. Similarly, Vegetal-inulin encapsulated bacteria showed a gradual decrease from an initial of 5.93 to 5.87 log_{10} cfu/ml and from 6.00 to 5.96 log_{10} cfu/ml within the first 30 min of exposure to SGF for VPC and FMA respectively (Fig 3.3). This was followed by an increase in cells to 6.43 and 6.7 log_{10} cfu/ml as per VPC and FMA respectively after 2 h (Fig 3.3). After exposure to SGF, results showed that encapsulation protected the cells as opposed to the unencapsulated cells. The number of cells released from Vegetal matrix after exposure to SGF were 0.90 and 0.78 log units as per VPC and FMA respectively, while the number of cells released from Vegetal-inulin matrix during same time frame were 0.60 and 0.75 log units by VPC and FMA respectively (Fig 3.3). This suggests that addition of inulin improved the effectiveness of Vegetal to protect bifidobacteria during exposure to SGF, thus encapsulation with Vegetal-inulin performed better at protecting the cells from gastric acidity.

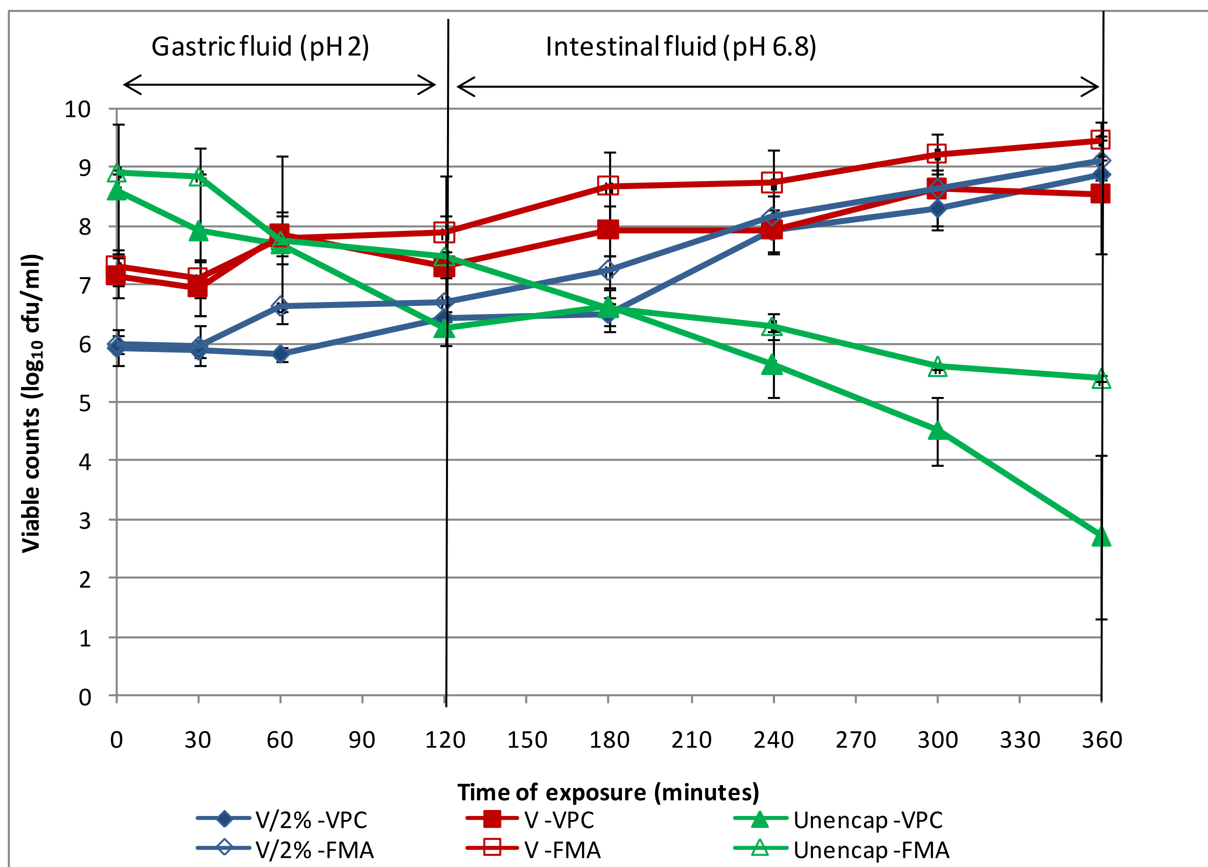

Figure 3.3 Survival of Vegetal BM 297 ATO-inulin encapsulated and unencapsulated *Bifidobacterium longum* LMG 13197 after exposure to simulated gastrointestinal fluids over 8 h. VPC= Viable plate count, FMA = fluorochrome microplate assay V= Vegetal, V/2% = Vegetal/ 2% inulin, Unencap = unencapsulated. Error bars represent standard deviation of means (n= 3).

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The numbers of the unencapsulated bacteria continued to decrease upon subsequent exposure to SIF (Fig 3.3). This resulted in a total bacterial cell loss of 5.89 and 3.52 log units as indicated by VPC and FMA respectively, after 8 h (Fig 3.3). Thantsha et al. (2009) also reported a continuous decrease in number of viable unencapsulated *B. longum* Bb-46. On the other hand, subsequent exposure of Vegetal encapsulated bacteria to SIF led to an increase in the number of viable bacteria, from an initial of 7.30 to 8.54 $\log_{10}$ cfu/ml and from 7.88 to 9.46 $\log_{10}$ cfu/ml at the end of 8 h for VPC and FMA respectively (Fig 3.3). This result suggests that at the high pH (6.8), the matrix dissolved and released cells into the fluid. Similarly, subsequent exposure of Vegetal-inulin encapsulated bacteria to SIF resulted in an increase in the number of viable bacteria cells, from an initial of 6.43 to 8.89 $\log_{10}$ cfu/ml and from 6.71 to 9.13 $\log_{10}$ cfu/ml after 8h as per VPC and FMA respectively (Fig 3.3). In support of our findings, Okuro et al. (2013) demonstrated that lipid microcapsules with prebiotics were disintegrated at pH 6.5 leading to the release of encapsulated bacteria.

Our results showed that between the two matrices, a high burst release of cells was observed immediately after suspension in SIF from Vegetal matrix (Fig 3.3). Burst releases of cells from Vegetal matrix were 0.62 and 0.79 log units as per VPC and FMA, respectively. The release from Vegetal-inulin matrix was lower, counted at 0.07 and 0.52 log units as per VPC and FMA, respectively (Fig 3.3). Although initial burst leads to higher cell delivery, it is not favourable as it can affect the ability of the matrix to maintain long term controlled release of cells (Huang and Brazel, 2001). Together, our results demonstrate that at the end of 8 h exposure to simulated gastrointestinal fluids, encapsulation provided significant protection to bifidobacteria cells as opposed the unencapsulated cells (Fig 3.3). Both Vegetal and Vegetal-inulin matrices showed continuous release of cells above 10^6 log cfu/ml in SIF, with no significant difference at the end of 8 h (Fig 3.3). Therefore, our results suggest that Vegetal-inulin matrix has the potential to release higher numbers of viable cells during gastrointestinal transit.

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3.4.2 Survival of encapsulated bacteria during storage

The results demonstrated that the number of unencapsulated bacteria was higher than the encapsulated bacteria when stored at 4ºC (Fig 3.4A). After 6 weeks of storage at 4ºC, the unencapsulated cells decreased from an initial count of 9.68 to 6.26 $\log_{10}$ cfu/g and from 9.73 to 6.3 $\log_{10}$ cfu/g as indicated by VPC and FMA, respectively (Fig 3.4A). Despite this decrease, unencapsulated cells were still present in sufficient amounts at the end of the 6 weeks. On the other hand, Vegetal encapsulated cells showed a decrease in cells from an initial of 8.28 to 5.17 $\log_{10}$ cfu/g and from 8.30 to 5.32 $\log_{10}$ cfu/g after 6 weeks of storage at 4ºC as per VPC and FMA, respectively (Fig 3.4A). Similarly, Vegetal-inulin encapsulated cells showed a decrease in cells from an initial of 8.60 to 5.16 $\log_{10}$ cfu/g and from 8.62 to 5.45 $\log_{10}$ cfu/g after 6 weeks of storage at 4ºC, indicated by VPC and FMA, respectively (Fig 3.4 A). These reductions in viable cells might suggest partial release of bacteria from the matrix. The results of this study were different from those of Fritzen-Freire et al. (2012), who reported that *B. lactis* BB-12 cells encapsulated with and without inulin remained high for 12 weeks. The reason for this discrepancy could be due to the fact that *B. lactis* has been known to survive extreme conditions than other *Bifidobacterium* strains (Ruiz et al., 2011). When compared to the unencapsulated cells, our results demonstrate that encapsulation did not offer any extension in shelf life of bifidobacteria cells at 4°C, as protection was only provided for 4 weeks (Fig 3.4 A). When compared to the Vegetal encapsulated cells, FMA showed that Vegetal-inulin matrix contained viable cells above 6 $\log_{10}$ cfu/g by the 5th week of storage (Fig 3.4 A).

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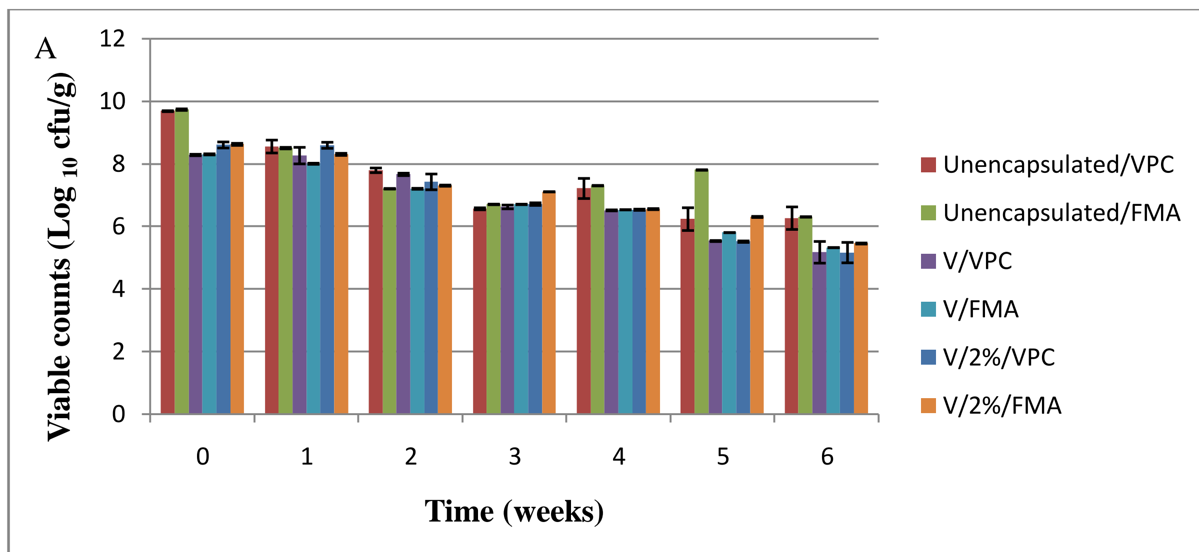

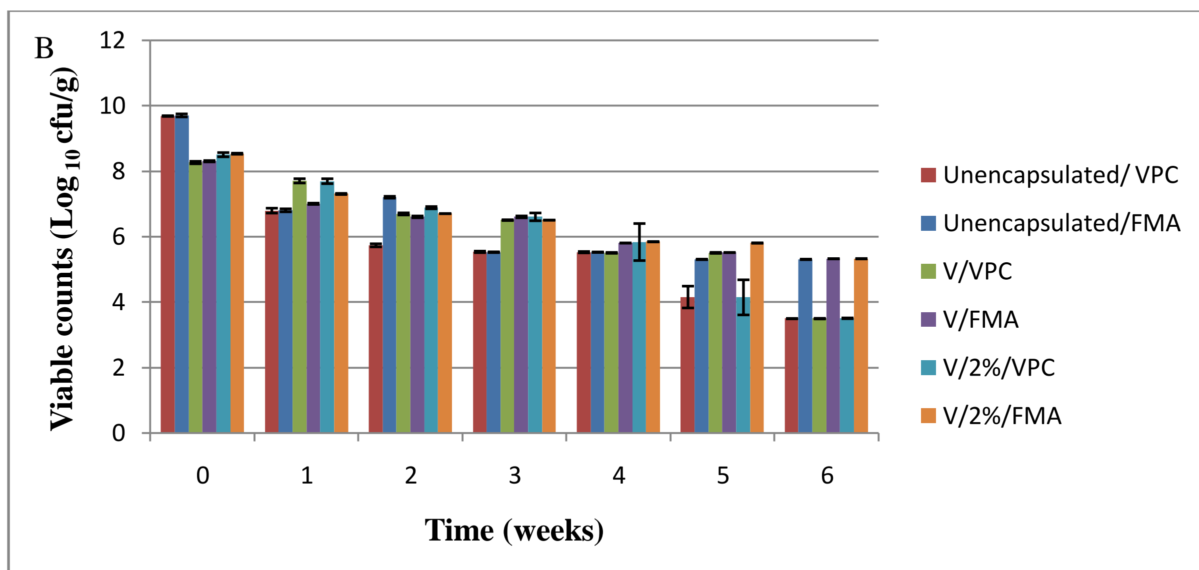

Figure 3.4 Survival of unencapsulated and encapsulated *B. longum* LMG 13197 during storage at (A) 4°C and (B) 25°C for 6 weeks. V/2% = Vegetal+2% inulin; V= Vegetal; VPC= viable plate count; FMA= fluorochrome microplate assay. Each bar represents the mean of triplicate data from three independent trials; error bars represent standard deviations.

Figure 3.4 B shows survival of *B. longum* LMG 13197 during 6 weeks of storage at 25°C. Samples stored at 25°C showed a rapid decrease in viability for unencapsulated cells than for the encapsulated bacteria (Fig 3.4B). Viable counts of unencapsulated cells decreased from initials of 9.68 to 3.49 $\log_{10}$ cfu/g and from 9.70 to 5.30 $\log_{10}$ cfu/g after 6 weeks of storage at 25°C for VPC and FMA, respectively (Fig 3.4B). These results demonstrate that numbers of viable unencapsulated bacteria stored at 25ºC dropped below the minimum amount of 10^6

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cfu/g after only 2 weeks (Fig 3.4B). On the other hand, Vegetal encapsulated bacteria showed a decrease in viable cells from initials of 8.26 to 3.49 $\log_{10}$ cfu/g and from 8.30 to 5.32 $\log_{10}$ cfu/g after 6 weeks of storage at 25ºC for VPC and FMA, respectively (Fig 3.4B). Similarly, Vegetal-inulin encapsulated bacteria showed a decrease in cells from initials of 8.50 to 3.50 $\log_{10}$ cfu/g and from 8.53 to 5.32 $\log_{10}$ cfu/g after 6 weeks of storage at 25ºC for VPC and FMA, respectively (Fig 3.4B). Our results demonstrated that the number of viable encapsulated bacteria (with and without inulin) dropped below 10^6 cfu/g after 3 weeks (Fig 3.4B). By the end of 6 weeks, VPC showed that counts of unencapsulated bacteria had decreased by 6.19 log units, while the encapsulated bacteria decreased by 4.77 and 5.00 log units for Vegetal and Vegetal-inulin matrices, respectively. When compared to the unencapsulated cells, our results demonstrate that encapsulation offered an increase in shelf life of bifidobacteria cells at 25ºC, with quality protection extended to 3 weeks (Fig 3.4 B), demonstrating an increase of shelf life by one week. There was no significant difference between the protections offered by either Vegetal or Vegetal-inulin matrix throughout storage period (Fig 3.4 B).

Oxygen toxicity has been reported as one of the main factors contributing to reduction in numbers of viable cells (Simpson et al., 2005). This can be attributed to an increase in lipid oxidation, which exposes the cells to deleterious oxygen levels (Simpson et al., 2005; Heidebach et al., 2010). To circumvent this problem, the use of vacuumed glass bottles as well as desiccators has been suggested as an alternative storage medium (Shah, 2000; Hsiao et al., 2004; Castro et al., 1995; Heidebach et al., 2010). Hsiao et al. (2004) reported better survival of *B. longum* Bb46 cells in glass bottles as compared to those in polyethylene terephthalate (PET) bottles, as glass bottles have less oxygen permeability. In this study, the encapsulated probiotics were not stored in a vacuum and the samples were continuously exposed to oxygen, during weekly sampling, suggesting that exposure to oxygen may have contributed to the loss in viability in glass bottles (Okuro et al., 2013). Other studies have reported on the increased survival of probiotics during storage using various encapsulation methods and matrices (Ann et al., 2007; Weinbreck et al., 2010; Okuro et al., 2013). Our results show that although an increase in cell loss was observed more at 25ºC, encapsulation using both Vegetal and Vegetal-inulin matrices improved survival of probiotics at 25ºC as opposed to the unencapsulated cells. On the other hand, encapsulation with Vegetal did not really increase shelf life of bacteria when compared to the unencapsulated cells at 4°C, but the addition of inulin offered better protection. This is the first study reporting the survival of

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B. longum encapsulated in lyophilized Vegetal-inulin lipid based synbiotic microparticles at different storage conditions. Our results suggest that most favourable shelf-life will be achieved if *B. longum* LMG 13197 encapsulated in Vegetal BM 297 ATO-inulin are stored under refrigerated conditions.

3.4.2.1 Survival of encapsulated bacteria during storage (flow cytometry)

Flow cytometry was used in combination with Live/Dead Baclight kit L7012 (Molecular Probes) to assess viability of unencapsulated and encapsulated *B. longum* LMG 13197. Viability was evaluated by quantifying the relative percentages of each subpopulation during storage at 4°C and 25°C for 6 weeks. Dot plots of green and red fluorescence were used to differentiate bacterial populations. Each population was represented in quadrants; Q_1-injured cells ($SYTO9^+$ PI^+); Q_2- live cells ($SYTO9^+$ PI^-); Q_3- unstained viable or lysed cells ($SYTO9^-$ PI^-); Q_4- dead cells (PI^+ $SYTO9^-$). Figure 3.5 shows the flow cytometric dot plots which represent controls used for compensation settings. These settings allowed for proper discrimination between live, damaged and dead cells by dual staining method. SYTO9 stains all the bacteria cells in a populations, whether dead or alive (Fig 3.5 B and D). PI stains only cells with damaged or compromised bacterial membrane (Fig 3.5 C and E). Dual staining of bacteria cells in a population shows how SYTO9 stains all the cells, but in the presence of PI, SYTO9 is displaced and stains only cells with compromised membranes (3.5 F) (Auty et al., 2001; Lahtinen et al., 2006).

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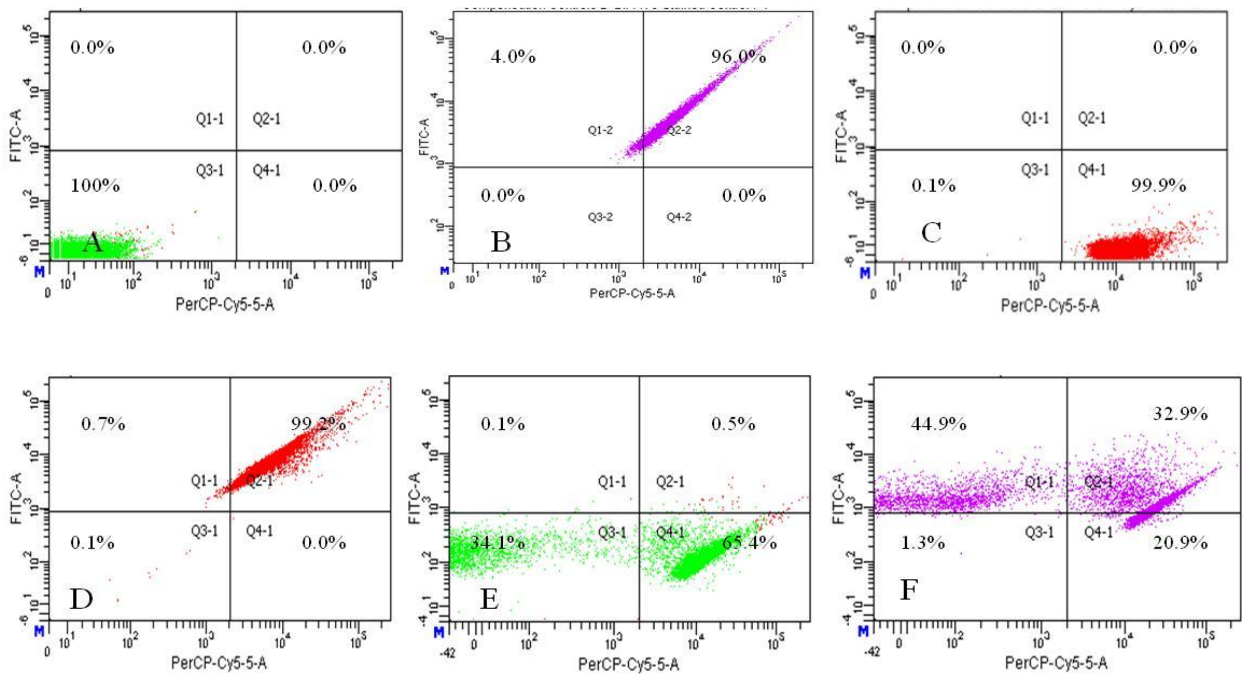

Fig 3.5 Dot plots showing setting of quadrants using *B. longum* LMG 13197 controls: A (Unstained cells), B (SYTO9 stained live cells), C (PI stained dead cells), D (SYT09 stained mixed cells), E (PI stained mixed cells) and F (Dual stained mixed cells).

The results demonstrated that the number of unencapsulated bacteria were high at initial stage of storage at 4ºC (Fig 3.6A). The number of live cells (Q_2) reduced from 63.8% to 14.5%, the injured cells (Q_1) increased from 34.0% to 59.7% and the dead cells increased from 1.9% to 7.4 % (Q_4) at 6th week of storage (Fig 3.6B). The increase in the number of injured cells suggests that over time, the cell membranes experienced an intermediate degree of damage which allowed the penetration of PI (Liu et al., 2007). However, this damage was not enough for them to be recorded as dead, as depicted by the low numbers of dead cells (Fig 3.6B). Light scatter pattern is indicative of bacteria size and granularity or complexity (Veal et al., 2000; Schenk et al., 2011). The results showed a diffuse scatter pattern for the unencapsulated bacteria throughout the storage period, suggesting that there were no significant changes to the size of the cells (Fig 3.6A and B). In correlation to the results obtained from VPC, FCA showed that the number of unencapsulated cells remained high by the 6th week of storage.

After 6 weeks of storage at 4°C, the population of Vegetal encapsulated bacteria showed a decrease of 7.2% for live cells, a decrease of 0.3% for injured cells and an increase of 19%

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for dead cells (Fig 3.6 C and D). In the same time frame, the population of Vegetal-inulin encapsulated bacteria showed a decrease of 1.8% for live cells and increases of 0.3% and 21.7% for injured and dead cells, respectively (Fig 3.6 E and F). The high bacterial numbers observed in the dead quadrants of encapsulated bacteria by the 6th week of storage at 4°C (Figs 3.6 C, D and 3.6 E, F), suggests damage to the bacterial membrane overtime, which is often associated with environmental stresses (Beal et al., 2001; Ananta et al., 2005; Cánovas et al., 2007; Rault et al., 2007; Martin-Dejardin et al., 2013). Previous studies have shown that freeze dried lactic acid bacteria exhibited more than 50% of dead cells after one month storage at -80°C (Rault et al., 2007). Light scatter pattern also demonstrated slightly dispersed bacteria patterns during the first week for both encapsulated samples (Fig 3.6 C and E). By the 6th week, the cell population light dispersion became more concentrated (Fig 3.6 D and F), indicating alterations in the size and internal structure of bacteria as reported by Hewitt et al., (1999). In correlation to the results obtained from VPC, FCA showed that the number of viable encapsulated cells decreased by the 6th week of storage, with a high percentage decrease in live cells for Vegetal encapsulated cells than Vegetal-inulin encapsulated cells.

The high percentage of unstained cells observed in Q_3 (Fig 3.6) could suggest that; 1) The stress associated with freeze drying reduced the ability of cells to take up SYTO9, 2) The bacterial membrane integrity was relatively high and PI could not penetrate across membrane and stain nucleic acids, 3) The presence of debris which are often seen in treated samples and 4) The presence of lysed bacteria that may have lost their nucleic acids and therefore cannot be stained (Hayouni et al., 2008; Martinez-Abad et al., 2012).

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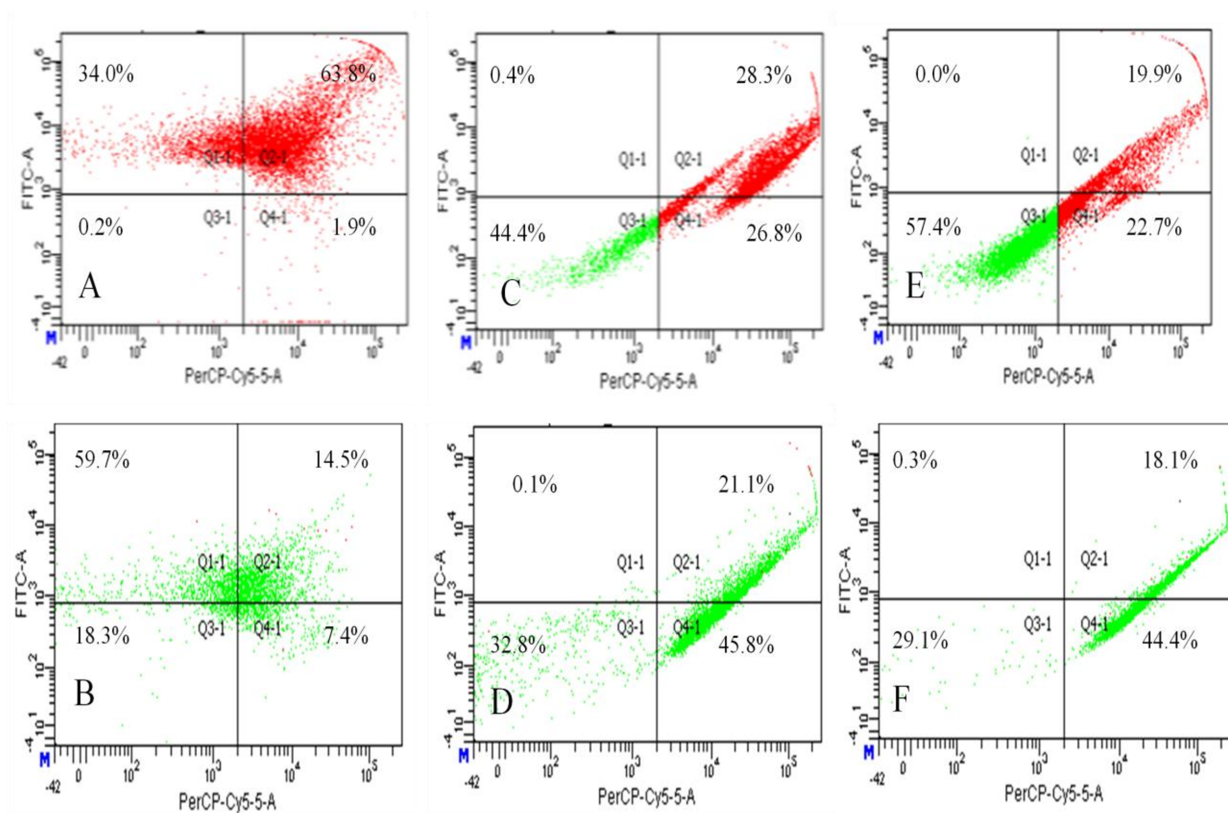

Fig 3.6 Dot plots showing the survival of *B. longum* LMG 13197 during storage at 4°C: unencapsulated at week 0 (A) and week 6 (B), Vegetal encapsulated at week 0 (C) and week 6 (D), Vegetal-inulin encapsulation at week 0 (E) and week 6 (F).

There was a drastic decline in the number of unencapsulated viable cells stored at 25°C after 6 weeks (Fig 3.7 A and B). Results showed a reduction of 51.6% and 31.2% for live and injured cells, respectively with a concomitant increase of 81.7% for dead cells after 6 weeks of storage at 25°C (Fig 3.7 A and B). By the 6th week, a decline in the number of Vegetal encapsulated viable cells stored at 25°C was also observed (Fig 3.7 C and D). For this sample, live cells decreased by 49.2%, while dead cells increased by 53% (Fig 3.7 C and D). Similarly, Vegetal-inulin encapsulated cells showed a 42.6% decline in the number of live cells and an increase of 46.8% for dead cells (Fig 3.7 E and F). Light scatter pattern demonstrated slightly dispersed bacterial populations during the first week (Fig 3.7 C and E) to more concentrated dispersion patterns six weeks post-storage (Fig 3.7 D and F) for both encapsulated samples. A concentrated bacterial dispersion pattern could suggest a decrease in bacterial size or alterations of cell morphology (Novik et al., 2001; Young et al., 2007; Schenk et al., 2011). In correlation to the results obtained from VPC, FCA clearly demonstrated a greater loss in bacterial viability at 25°C for all the samples analyzed, but the

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number of viable unencapsulated cells decreased more than the encapsulated cells by the 6th week of storage.

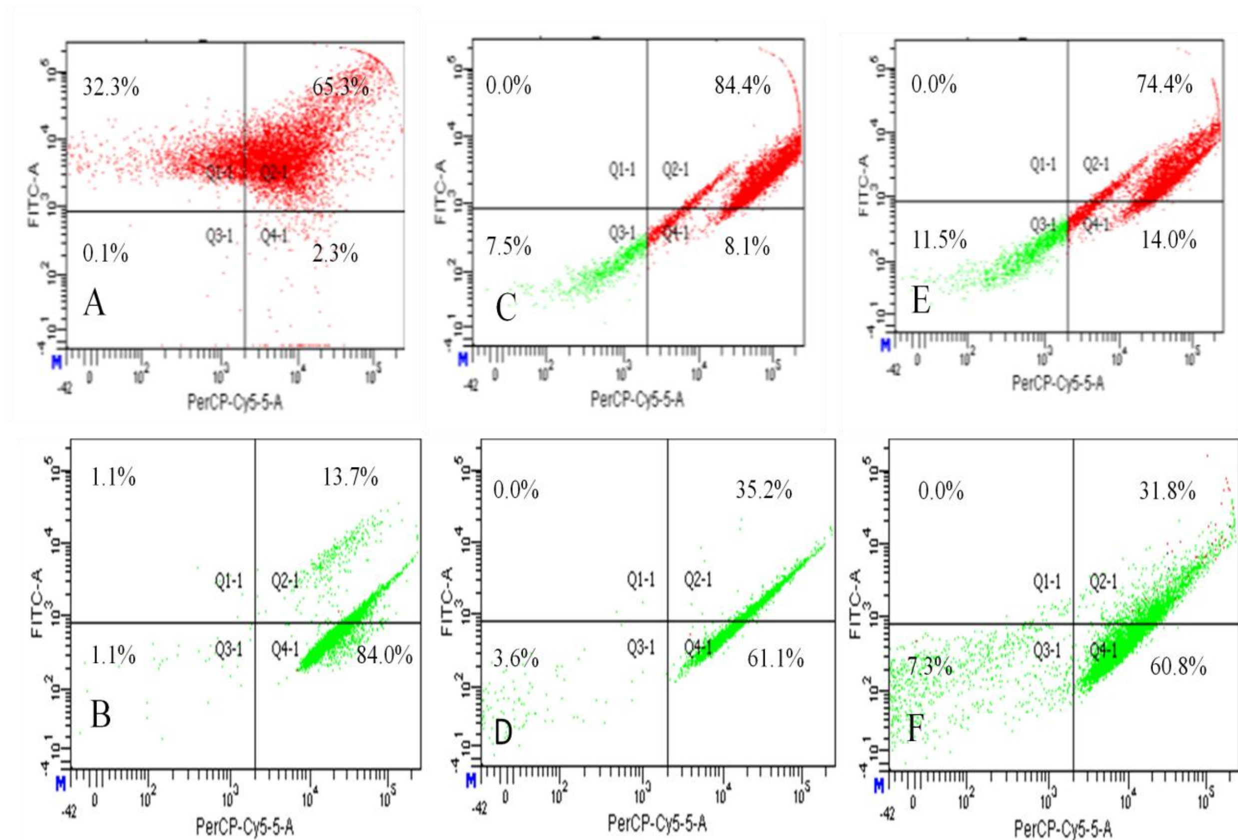

Fig 3.7 Dot plots showing the survival of *B. longum* LMG 13197 during storage at 25°C: unencapsulated at week 0 (A) and week 6 (B), Vegetal encapsulated at week 0 (C) and week 6 (D), Vegetal-inulin encapsulation at week 0 (E) and week 6 (F).

In summary, our results demonstrate that encapsulation proved essential during storage at 25°C, as encapsulated cells showed better survival than the unencapsulated cells. However, all the samples showed increased loss in viable cells at 25°C more than at 4°C by the end of storage period. Although the unencapsulated cells survived throughout the storage period at 4°C, Vegetal-inulin matrix seemed to offer better protection with a 1.8% decrease in live cells as opposed to a 7.2% decrease for Vegetal encapsulated cells. Therefore, in correlation with results obtained from VPC and FMA, optimal shelf life will be achieved under refrigerated conditions.

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3.5 Conclusion

The production of freeze dried lipid microparticles using Vegetal for the encapsulation of probiotics, offered protection during exposure to simulated gastrointestinal fluids. The presence of inulin improved the protection efficiency of Vegetal, as *B. longum* LMG 13197 cells were protected from gastric juice acidity and then released in sufficient amounts in SIF for colonization. In comparison to the unencapsulated cells, encapsulation improved the survival of the probiotics during 6 weeks of storage at 25°C. Although encapsulation did not significantly improve survival of probiotics during 6 weeks of storage at 4°C, Vegetal-inulin matrix offered considerable protection until the fifth week. Therefore, Vegetal-inulin microparticles have the potential to be applied as food adjuncts to readily consumable foods.

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CHAPTER 4

Survival of Vegetal-inulin encapsulated *Bifidobacterium longum* LMG 13197 in yoghurt and its influence on the physico-chemical attributes of yoghurt

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4.1 Abstract

Maintaining the viability of probiotics in yoghurt has become very challenging due to post acidification, which occurs during refrigeration storage. Consumers can only obtain the health benefits from yoghurt if the incorporated probiotics remain above the recommended therapeutic standard prior to consumption. Microencapsulation is expected to improve the survival of probiotics during this post acidification period. This study aimed at analysing viability of *Bifidobacterium longum* LMG 13197 encapsulated with Vegetal BM 297 ATO (with or without inulin) in yoghurt during six weeks of refrigeration storage. The change in pH and colour of yoghurt samples was monitored throughout the storage period following the incorporation of the microparticles. The number of viable cells was analyzed weekly using plate count method, microplate fluorochrome assay and flow cytometry. Viability analysis showed that the levels of Vegetal-inulin encapsulated *B. longum* LMG 13197 remained well above recommended therapeutic minimum (10^6 cfu/ml) at the end of the storage period, as opposed to the unencapsulated cells. The pH of yoghurt samples with Vegetal and Vegetal-inulin encapsulated probiotic showed no significant decrease ($p>0.05$), while yoghurt with unencapsulated cells decreased. There was a significant effect ($p<0.05$) on the lightness (L^*) and yellowness (b^*) of the yoghurt sample containing Vegetal-inulin encapsulated cells by the 6th week of storage and the total colour change was negligible. Therefore, Vegetal BM 297 ATO-inulin matrix protected *B. longum* LMG 13197 in yoghurt throughout storage period, with no increase in pH or negative effect to the appearance of yoghurt.

Keywords: Probiotics, inulin, yoghurt, Vegetal BM 297 ATO, *Bifidobacterium longum* LMG 13197, fluorochrome microplate assay, flow cytometry, microencapsulation.

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4.2 Introduction

One of the important developments in today's food industries is the production of functional foods and functional food supplements for health purposes (Saarela et al., 2002). In light of this, probiotics have been used to improve health of individuals (Venter, 2007; Fooks and Gibson, 2002; Iyer and Kailasapathy, 2005). Probiotics are defined as"live microorganisms which when taken in adequate amounts confer beneficial effects to the host" (FAO/WHO, 2002). The increasing need for improved health values led to incorporation of probiotics into fermented dairy products, such as yoghurt (Venter, 2007). Yoghurt is an acidic fermented dairy food, which is consumed worldwide and has become an important vehicle by which consumers can ingest probiotic cells (Sun and Griffiths, 2000). The acidic constituent of yoghurt is as a result of the presence of starter cultures (*Streptococcus salivarius subsp thermophilus* and *Lactobacillus delbrueckii subsp bulgaricus*) (Younus et al., 2002). The starter cultures bring about fermentation which gives yoghurt its unique taste, texture, colour and odour. However, these unique characteristics can be negatively affected if the metabolites produced by starter cultures are not limited, and can lead to rejection by the consumers and increased economic losses (Arai et al., 1996; Aswal et al., 2012). Therefore, the organoleptic and physico-chemical properties of yoghurt must be analysed to ensure that its quality remains acceptable to consumers (Gonzalez et al., 2011).

In addition, consumers have to be assured of continuous nutritional availability as well as an acceptable shelf life. Hence, it is essential to ensure that the probiotic cells remain viable and in sufficient amounts of 10^6-10^7cfu/ml or g in this probiotic product in order to provide the required health benefits (Lee and Salminen, 1995; Özer et al., 2005). These requirements are met by performing shelf life and survival studies of the probiotics present in yoghurt (Bunthof et al., 2001; Bunthof and Abee, 2002; Picot and Lacroix, 2004; Iyer and Kailasapathy, 2005; Lahtinen et al., 2006; Vasiljevic and Shah, 2008). Probiotic strains such as *Bifidobacterium lactis* and *B. longum* have been increasingly incorporated into yoghurt, because they have been reported to tolerate acidity (Sun and Griffiths, 2000; Akalin et al., 2004; Takahashi et al., 2004; Shah, 1997). It has been reported that addition of these probiotics into yoghurt improved its nutritional as well as therapeutic values (Burgain et al., 2011). However, several studies have reported a decrease in the number of viable bifidobacteria in yoghurt due to factors which include oxygen, temperature and post-

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production acidification brought on by decrease in pH after fermentation and during refrigeration storage (Lankaputhra and Shah, 1995; Lourens-Hattingh and Viljoen, 2001; Lee and Lucey, 2010).

Over the years, the addition of prebiotics as well as the use of microencapsulation has been essential for the improved survival and viability of probiotic strains in yoghurt (Gallaher and Khil, 1999; Sultana et al., 2000; Sun and Griffiths, 2002; Akalin et al., 2004; Iyer and Kailasapathy, 2005; Özer et al., 2005; Capela et al., 2006; Aryana and Mcgrew, 2007). It is of importance that the materials used for encapsulation be approved by the Food and Drug Administration (FDA) as a food grade material (Burgain et al., 2011), they must also be able to protect the cells from acidic stresses and subsequently release viable cells for improved health (Pimentel-González et al., 2009). However, the delivery of lipid based synbiotic microparticles encapsulating probiotics through yoghurt for beneficial purposes has not been much explored. This study aimed to assess the survival of Vegetal BM 297 ATO-inulin encapsulated *B. longum* LMG 13197 when incorporated into yoghurt and the effect of the microparticles on the physico-chemical properties of yoghurt.

4.3 Materials and Methods

4.3.1 Reagents and bacterial cultures

Biogapress Vegetal BM 297 ATO was obtained in powdered form from (Gattefossé SAS). *Bifidobacterium longum* LMG 13197 cultures were obtained from BCCM/LMG Culture collection (Belgium) as 20% glycerol stocks in MRS broth at -70°C and revived according to the manufacturer's specifications. Inulin (purity: 95%), polyvinyl alcohol (PVA) 87-89% partially hydrolysed (Mw: 13000-23000 Da), lactose monohydrate (purity: 99%) were obtained from Sigma Aldrich, South Africa, while dichloromethane (DCM) (analytical grade, purity: 99%) was obtained from Sigma Aldrich Laborchemikalien, Seelze.

4.3.2 Bacterial culturing and enumeration

Frozen stocks of *B. longum* LMG 13197 were allowed to thaw and streaked out onto MRS-cys-HCl agar plates and incubated in anaerobic jars with Anaerocult A gaspaks and

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Anaerocult C strip for 48 h at 37°C to obtain single colonies. A single colony was then inoculated into 5 ml MRS-cys-HCl broth and incubated anaerobically overnight at 37°C. One millilitre of the overnight culture was then inoculated into 100 ml of MRS broth supplemented with 0.05% cysteine-HCl and incubated anaerobically at 37°C overnight. This culture was subsequently subcultured into fresh 100 ml MRS-cys-HCl broth and incubated as previously described. One millilitre of the culture was used for bacteria enumeration after dilution in Ringer's solution. A subsample of 100 µl was taken from the broth culture and suspended in 900 µl of ¼ strength Ringer's solution. A 10-fold serial dilution was performed and cells were plated out onto MRS-cys-HCl agar plates in triplicates and plates were incubated (General purpose incubator, Shel Lab) at 37°C for 72 h under anaerobic conditions. After incubation, the number of viable cells were determined and recorded as cfu/ml. The remaining 3 ml of the culture was used for bacterial encapsulation while the rest was stored in 10% sterile glycerol at -20°C.

4.3.3 Bacteria encapsulation

Encapsulation method was done according to Pimentel-Gonzalez et al. (2009) with modification. The stored *B. longum* LMG 13197 cultures was allowed to thaw and 1 ml was subcultured into three 250 ml flasks containing 100 ml MRS-cys-HCl broth, and incubated anaerobically at 37°C for 48 h. After incubation, bacteria were harvested by centrifugation, using an Eppendorf centrifuge 5804R (cooled to 4°C) at 20 800 g for 15 min. The pelleted cells (approximately 3.14×10^{8} cfu/ml) were washed once with Ringer's solution and kept at 4°C for 5 min before encapsulation. The first emulsion was prepared by suspending the bacterial pellet into 1 ml of 2% (w/v) inulin. The bacteria-inulin mixture was then added to 1 ml of 2% (w/v) poly-vinyl-alcohol (PVA). The resulting suspension was subsequently added to 10 ml dichloromethane (DCM) containing Vegetal BM 497 ATO at a final concentration of 10% (w/v). The resulting emulsion was homogenized at 8000 rpm for 5 min using a Silverson, L4R, NIMR homogenizer and left to stand at 25°C. The second emulsion was prepared by mixing 15 ml of PVA 2% (w/v) and 5 ml of lactose 5% (w/v). The first emulsion was mixed into the second emulsion and homogenised at 8000 rpm for 5 min using a Silverson, L4R, NIMR homogenizer. The stable emulsion was left to stand in the fume hood for 5 h for DCM evaporation. After evaporation of DCM, the sample was frozen at - 20°C overnight. This was followed by freeze drying using a Virtis bench top, SLC, freeze dryer for

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3 days at -75°C. The freeze dryer was set at a condenser temperature and vacuum pressure of – 60°C and 0.26 millitor, respectively.

The same protocol was used to prepare Vegetal BM 297 ATO microparticles encapsulating *B. longum* LMG 13197 without inulin, except bacterial pellet was re-suspended in 1 ml of deionised water before mixing with 1 ml of 2% (w/v) poly-vinyl-alcohol (PVA). The control sample (unencapsulated cells) was prepared by re-suspending B. *longum* cells (approximately 3.14×10^8 cfu/ml) into 25 ml of sterile deionised water and fast-frozen in liquid nitrogen. The frozen cultures were frozen at – 70°C for 1 h before freeze drying in a Virtis bench top, SLC, freeze dryer for 3 days at -75°C. After freeze drying, all the samples were stored in tightly sealed sterile Schott bottles at 4°C for 1 h until further analysis.

4.3.4 Determination of total bacteria encapsulated

The encapsulated sample (0.1 g) was suspended in 900 µl of DCM and vortexed for 30 s. Mixture was spun down using a MiniSpin, Eppendorf at 12 100 g for 30 s and the supernatant removed. The pellet was then resuspended in 900 µl of ¼ strength Ringer’s solution. A tenfold serial dilution was performed and 100 µl of each dilution was plated out in triplicate onto MRS-cys-HCl agar using the pour plate method. The plates were incubated at 37°C for 72 h in anaerobic jars with Anaerocult A gaspaks and Anaerocult C test strip to indicate anaerobic conditions.

4.3.5 Survival of encapsulated *B. longum* LMG 13197 in yoghurt

4.3.5.1 Preparation of yoghurt

Yoghurt was prepared in the laboratory as follows: Two hundred and fifty millilitres of skimmed milk (Long life, First choice, South Africa) was mixed with one tablespoon (approximately 8 g) of powdered milk (NIDO, Nestle, South Africa) in three flasks. The mixture was homogenised by thoroughly swirling the flask and then pasteurised at 72°C for 3 min. The pasteurized milk was cooled to 42°C and inoculated with spoonful of plain yoghurt containing (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp *bulgaricus*). The

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inoculated milk was then incubated at 37°C until a pH of 4.53 was attained. Yoghurt was pasteurized at 72°C for 30 min to kill the starter cultures as previously done by Sun and Griffiths (2000). This was done to ensure that no viable lactic cultures remained before inoculating with bifidobacteria cells. One millilitre of yoghurt sample was suspended in 9 ml of ¼ strength Ringer's solution. A tenfold serial dilution was performed and 100 µl of each dilution was pour plated in triplicate onto M-17 and MRS-cys-HCl agar for enumerating *S. thermophilus* and *L. delbrueckii* subsp *bulgaricus*, respectively. The plates were incubated at 37°C for 72 h in anaerobic jars with Anaerocult A gaspaks and Anaerocult C test strip to indicate anaerobic conditions.

Then 30 ml of the fermented yoghurt was poured into 50 ml sterile containers. Each of the 30 ml aliquot of yoghurt was aseptically supplemented with 1 g of either unencapsulated or encapsulated *B. longum* LMG 13197 cells (with or without inulin) and stored at 4°C for 6 weeks. The viability of bifidobacteria cells was determined by taking subsamples of 1 ml once a week for bacterial enumeration using viable plate count (VPC), fluorochrome microplate assay (FMA) and flow cytometry assay (FCA). The '0 day' analysis was carried out after overnight cold storage of samples.

4.3.5.2 Enumeration of *B. longum* cells after storage in yoghurt

One millilitre subsamples obtained weekly were serially diluted (tenfold) in ¼ strength Ringer's solution. Aliquots of 100 µl from each dilution were plated out in triplicates onto MRS-cys-HCL agar supplemented with 0.05 % cysteine hydrochloride using the pour plate method. The plates were incubated at 37°C for 72 h under anaerobic conditions (Anaerobic jars with Anaerocult A gaspaks and Anaerocult C test strip). The number of colonies observed were counted, calculated and reported as colony forming units per millilitre (cfu/ml).

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4.3.6 Fluorochrome microplate assay

4.3.6.1 Preparation of standard curve

The standard curve preparation was set up as follows: One hundred microlitres of *B. longum* LMG 13197 was inoculated into 25 ml of MRS broth supplemented with 0.05 % cysteine hydrochloride, and then incubated in a shaker incubator at 37°C in anaerobic jars with Anaerocult A gaspaks and Anaerocult C test strips for 72 h. Bacteria from this culture were harvested by centrifugation using the Eppendorf centrifuge 5804R (cooled to 4°C) at 20 800 g for 15 min. The pellet obtained was re-suspended in 2 ml of 0.85 % NaCl in a Falcon tube. One milliliter of the suspension was mixed with 20 ml of 0.85 % NaCl in two separate Falcon tubes labeled live and dead. Sample labeled dead was heat treated in a microwave for 2 min to kill the bacteria. Both live and dead bacteria samples were centrifuged using the Eppendorf centrifuge 5804R (cooled to 4°C) at 20 800 g for 15 min to pellet the cells. Pellets were re-suspended in 10 ml of 0.85 % NaCl. The cultures were adjusted to optical density at 600 nm (OD_{600}) of ~0.4 using a Spectronic® 20 Genesys™ spectrophotometer. Different volumes of live and dead bacteria (Table 4.1) were mixed to achieve different proportions of live: dead cells for determining a standard curve. Viable bacterial counts were determined by VPC for each bacterial mixture and used to correlate the green-to-red ratio obtained after staining. These values were used to plot the standard curve.

Table 4.1: Proportions of live: dead cells used to calculate standard curve.

Ratio of live : dead cells	Live cell suspensions (ml)	Dead cell suspensions (ml)
0:100	0	2.0
10:90	0.2	1.8
50:50	1.0	1.0
90:10	1.8	0.2
100:0	2.0	0

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4.3.6.2 Staining of bacteria suspensions and fluorochrome microplate assay

The staining of bacteria cultures was performed using a LIVE/DEAD ® Baclight ™ bacterial viability kit. The bacterial viability kit utilizes propidium iodide (PI) (red fluorescence at 620 nm) and SYTO9 (green fluorescence at 530 nm). The staining solution was prepared according to the manufacturer's instructions to obtain 3.34 mM of SYTO9 and 20 mM of PI stock solutions dissolved in dimethyl sulfoxide. From these concentrations, a 2x staining solution was made by mixing 6 µl of PI and 6 µl of SYTO9 in 2.0 ml of filter sterilized water. Bacteria suspensions were then stained by mixing 100 µl of the bacterial suspension (approximately 1×10^9 cfu/ml) with 100 µl of the 2x staining solution in triplicates into a black 96 well fluorescence microtitre plate, as previously described (Alakomi et al., 2005). The microtitre plates were covered with foil and incubated in the dark for 15 min at room temperature. Fluorescence of bacterial suspensions was measured using the automated Fluorometer Fluoroskan Ascent FL (Lab systems, Helsinki, Finland). The excitation and emission of red fluorescence were 530 nm and 635 nm respectively, while those of green fluorescence were 485 nm and 538 nm respectively. The intensities of the green-to-red ratio was used to compute the colony forming unit in each sample based on the standard curve previously prepared for *B. longum* LMG 13197 (Fig 4.1).

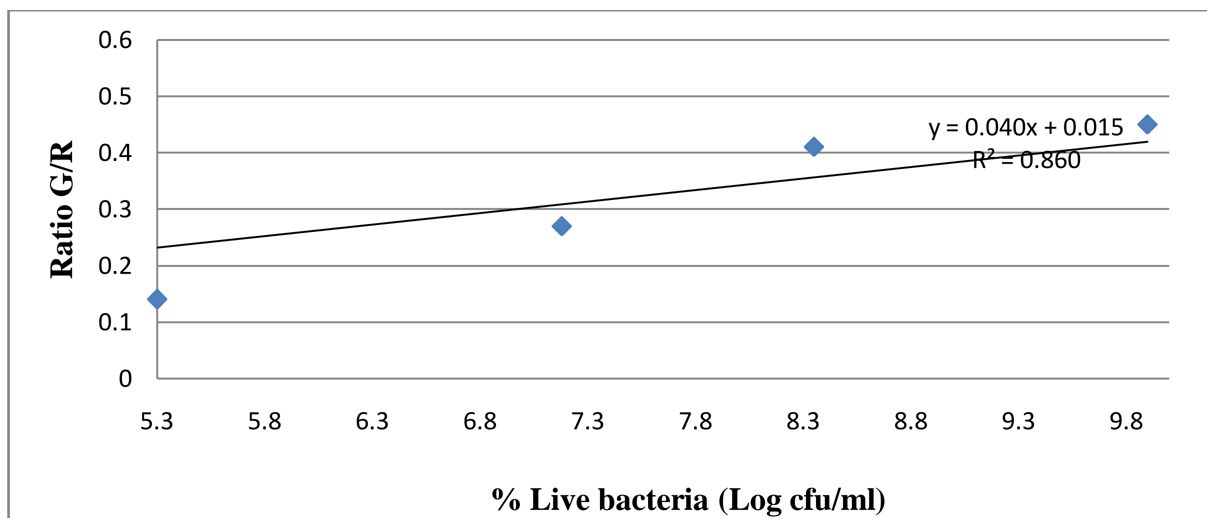

Fig 4.1 Standard curve for *B. longum* LMG 13197 showing relationship between proportion of live bacteria (log cfu/ml) and green/red fluorescence ratio (Ratio $_{G/R}$).

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4.3.6.3 Fluorochrome microplate assay of *B. longum* LMG 13197 cells after storage in yoghurt

One millilitre of unencapsulated bacteria obtained after storage in yoghurt was suspended in 9 ml of ¼ Ringer's solution, vortexed for 30 s and pellets were obtained by centrifugation at 12 100 g for 5 min. One millilitre of encapsulated bacteria was suspended in DCM to disrupt the lipid matrix and release the bacteria. Samples were centrifuged at 12 100 g for 5 min. Pellets were re-suspended in 9 ml of ¼ Ringer's solution and centrifuged at 12 100 g for 5 min. Pellets obtained from the unencapsulated and encapsulated samples were then re-suspended in 0.85 % NaCl (Alakomi et al., 2005). The bacterial suspensions were adjusted to optical density at 600 nm (OD_{600}) of ~0.4 using a Spectronic® 20 Genesys™ spectrophotometer. Staining was performed by mixing 100 µl of the bacterial suspension with 100 µl of the 2x staining solution (6 µl of PI and 6 µl of SYTO9 in 2.0 ml of filter sterilized water) in triplicates into a black 96 well fluorescence microtitre plate. The microtitre plates were covered with foil and incubated in a dark cupboard for 15 min at room temperature. Fluorescence of bacterial suspensions was measured using the automated Fluorometer Fluoroskan Ascent FL (lab systems, Helsinki, Finland) as previously described.

4.3.7 Flow cytometry assay

4.3.7.1 Preparation of standard curve

One hundred microlitres of *B. longum* LMG 13197 was grown in 25 ml of MRS broth supplemented with 0.05 % cysteine hydrochloride and incubated in a shaker incubator at 37°C under anaerobic conditions for 72 h. Twenty five milliliter of this culture was harvested by centrifugation at 20 800 g for 15 min. The pellet obtained was re-suspended in 2 ml of phosphate buffered saline (PBS) in a Falcon tube. One milliliter of the suspension was mixed with 20 ml of PBS in two separate Falcon tubes labeled live and dead. Sample labeled dead was heat treated in a microwave for 2 min to kill the bacteria. Both Live and dead samples were centrifuged at 20 800 g for 15 min to pellet the bacterial cells. The resulting pellets were re-suspended in 10 ml of PBS and the OD_{600} adjusted to ~0.4 using a spectrophotometer (Spectronic® 20 Genesys™). Different volumes of live and dead bacteria were mixed and

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used to determine the standard curve as indicated in Table 4.1. Viable counts was determined for each bacterial suspension and used to correlate the percentage of live bacteria obtained from flow cytometry to plot the standard curve (Fig 4.2).

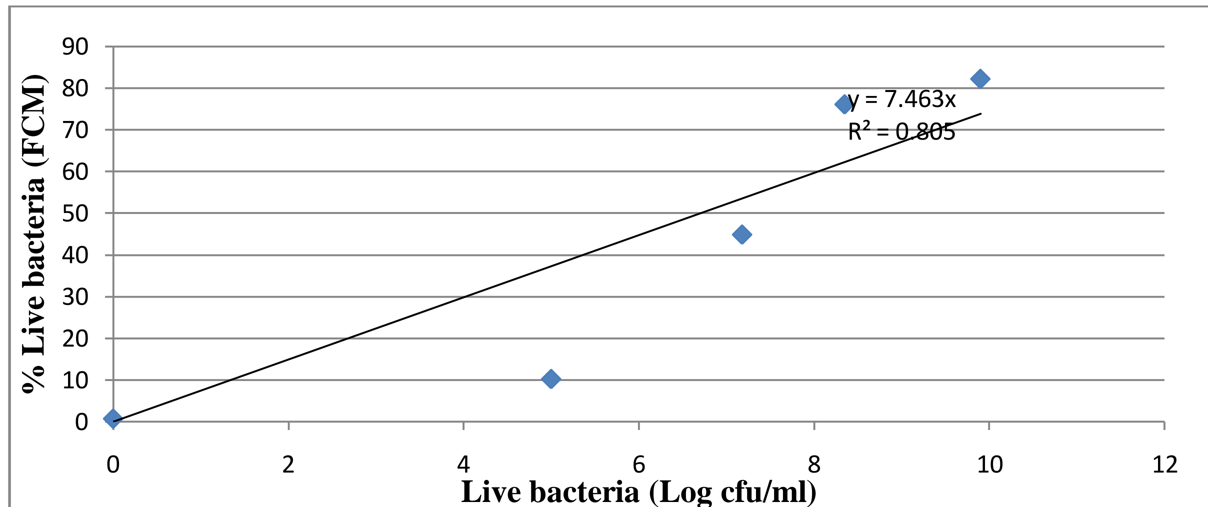

Fig 4.2 Standard curve for *B. longum* LMG 13197 showing relationship between proportion of live bacteria (log cfu/ml) and measured FCM live cells.

4.3.7.2 Staining of bacteria suspensions and flow cytometry assay

Staining of bacteria cultures was performed in triplicates using Live/Dead BacLight bacterial viability kit L7012 (Molecular Probes). Hundred microliters of the bacterial suspension (approximately 10^9 cfu/ml) was mixed with 100 µl of staining mix (1.5 µl of SYT09, 1.5 µl of PI and 1 ml of filter sterilized water) into Eppendorf tubes. The tubes were covered with foil and kept in a dark cupboard for 15 min. After incubation, the samples were transferred to a glass cuvette for analysis using a flow cytometer (FAC-Scan flow cytometer, Becton-Dickinson, Le Pont de Claix, France). The FAC-Scan analyzes cells passing through the sheath fluid using five band filters which include forward angle light scatter (FSC), side angle light scatter (SSC) and 3 fluorescence signals. These signals recognise fluorescent colours at different wavelengths and they include 530 nm {collects green fluorescence of SYT09, 30 nm band width, band pass filters ranging from 675- 715 nm (FITC)}, 585 nm {collects yellow-orange fluorescence} and 695 nm {collects red fluorescence of PI, 40nm band width, band pass filters ranging from 515-545nm (Per-C-P)}. FACSDIVA Version 6.1.3 was used to

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perform data analysis and the spectral overlap between the fluorescences emitted by both stains was removed by adjusting the compensation values and voltage threshold.

4.3.7.3 Preparation of control samples

One hundred microliters of *B. longum* LMG 13197 was grown in two flasks containing 25 ml of MRS broth supplemented with 0.05 % cysteine hydrochloride and incubated in a shaker incubator at 37°C under anaerobic conditions for 72 h. After incubation, the flasks containing bacteria cultures were labelled live and dead. Sample labeled dead was heat treated in a microwave for 2 min to kill the bacteria. One millilitre subsample of the live sample was transferred into two Eppendorf tubes and labelled live and unstained, respectively, while 1 ml subsample of the dead sample was transferred into an Eppendorf tube and labelled dead. These samples represented the controls used to differentiate the various bacterial populations into quadrants and to calculate the compensation of spectral overlap of different fluorochromes. The Eppendorf tubes containing live, dead and unstained samples were centrifuged at 20 800 g for 15 min to pellet the bacterial cells. The resulting pellets were re-suspended in 1 ml of PBS and the OD_{600} adjusted to ~0.4 using a spectrophotometer (Spectronic® 20 Genesys™). The staining of bacteria cultures was performed in triplicates using Live/Dead BacLight bacterial viability kit L7012 (Molecular Probes). The Eppendorf tubes containing live and dead bacterial cells were stained separately with 1.5 µl of SYT09, 1.5 µl of PI and 100 µl of mixed stains (1.5 µl of SYT09, 1.5 µl of PI and 1 ml of filter sterilized water). After staining the cells, the tubes were covered with foil and kept in a dark cupboard for 15 min before performing flow cytometry analysis as described in section 4.3.7.2.

4.3.7.4 Flow cytometry assay of cells after storage in yoghurt

One millilitre subsample of unencapsulated bacteria obtained after survival in yoghurt was suspended in 9 ml of ¼ Ringer's solution, vortexed for 30 s and centrifuged at 12 100 g for 5 min to obtain pellets. One millilitre subsamples of encapsulated bacteria were suspended in DCM and vortexed for 30 s to disrupt the encapsulating matrix and release the bacteria. The suspensions were centrifuged at 12 100 g for 5 min. The pellets obtained were re-suspended

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in 9 ml of ¼ Ringer's solution and centrifuged at 12 100 g for 5 min. Pellets obtained from the unencapsulated and encapsulated samples were then re-suspended in 1 ml of PBS. The bacterial suspensions were adjusted to optical density at 600 nm (OD_{600}) of ~0.4 using a Spectronic® 20 Genesys™ spectrophotometer. Dual staining was performed in triplicates with Live/Dead BacLight bacterial viability kit L7012 (Molecular Probes), where the bacterial suspensions were stained with 100 µl of (1.5 µl of SYT09, 1.5 µl of PI and 1 ml of filter sterilized water). After staining the cells, the tubes were covered with foil and incubated in a dark cupboard for 15 min before flow cytometry analyses as previously described.

4.3.8 Physico-chemical analysis of yoghurt

4.3.8.1 pH analysis

The pH of 9 ml subsample taken from the inoculated yoghurt was measured weekly throughout 6 weeks of storage at 4°C using a Crison Basic 20 pH meter (Denver instruments, USA). Measurements were done in triplicates and the average was recorded.

4.3.8.2 Colour analysis

A Minolta Chroma Meter CR-400 (Konica Minolta, Osaka, Japan) calorimeter was used to analyse colour of 9 ml subsample taken from the inoculated yoghurt throughout 6 weeks of storage at 4°C as described by De Castro-Cislaghi et al. (2012). The yoghurt samples were poured into a sterile petri dish and triplicate readings were taken from each yoghurt sample. The CIELab colour scale measured the following parameters; L* (ranging from 0 to 100, which represents variations from black to white), a* (represents variations from red+ to green–) and b*(represents variations from yellow+ to blue–). The total colour difference (ΔE*) between the unencapsulated sample and each of the encapsulated samples was calculated using the equation below as described by Fritzen-Freire et al. (2012).

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

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4.3.9 Statistical analysis

Mean values and standard deviations were calculated from the data obtained from three independent trials. Data was analysed using one way ANOVA (analysis of variance), the difference between the means was calculated using LSD (Least Significant Difference) and a p-value less than 0.05 was considered to be statistically significant. Analysis was performed using Statistica version 6.0 (Statsoft Inc, Tulsa, USA).

4.4 Results and Discussions

4.4.1 Survival of encapsulated bacteria in yoghurt as determined by VPC and FMA

Post fermentation acidification has been shown to decrease the viability of probiotics in yoghurt during refrigeration storage (Cruz et al., 2009; Rajapaksha et al., 2013). It is important that probiotic cultures be maintained in yoghurt at levels equal to or above the recommended therapeutic standard for probiotics (10^6 - 10^7 cfu/ml) prior to consumption (Bhadoria and Mahapatra, 2011). Therefore, microencapsulation should ideally protect the probiotic cells from post fermentation acidification.

Our results showed that the unencapsulated cells decreased from 8.52 to 5.82 $\log_{10}$ cfu/ml (2.70 log units loss) and from 8.54 to 5.90 $\log_{10}$ cfu/ml (2.64 log units loss) as indicated by VPC and FMA, respectively, during storage in yoghurt after six weeks (Fig 4.3). The decrease in viable unencapsulated cells was not significant until the sixth week, when their level dropped below 6 $\log_{10}$ cfu/ml (Fig 4.3). This non-significant decrease in the unencapsulated cells can be attributed to the absence of the starter cultures, which would have increased the levels of acid production in their presence (Samona et al., 1996). Although it has been reported that probiotics alone produce low levels of acid (Samona et al., 1996), this study suggests that in the absence of encapsulation, continued exposure of bifidobacteria cells to lactic acids during the storage period possibly led to their death (Kailasapathy, 2006; Burgain et al., 2011). Another possible reason for the decrease in unencapsulated cells is the presence of oxygen which was likely introduced during the production and storage of yoghurt (Miller et al., 2002). It has been reported that probiotics

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lack oxygen scavenging cellular mechanisms such as catalases. Thus, exposure of probiotics to oxygen leads to oxidative damage and subsequent death (Talwalkar and Kailasapathy, 2004).

Conversely, our results demonstrate a continuous increase in the number of viable bacteria in yoghurt samples containing encapsulated bacteria (Fig 4.3). Our results showed that although there was a similar trend between FMA and VPC data, the FMA results gave higher counts than those obtained for VPC ($p<0.05$) (Fig 4.3). Viable counts of cells encapsulated with Vegetal increased from 5.62 to 6.54 $\log_{10}$ cfu/ml (0.89 log increase) and from 6.00 to 7.10 $\log_{10}$ cfu/ml (1.10 log increase) as per VPC and FMA, respectively, after six weeks of storage in yoghurt (Fig 4.3). There was no significant difference in the number of cells released from Vegetal matrix until the fifth week of storage (Fig 4.3). The cells encapsulated with Vegetal-inulin showed a similar trend, with increase in cells from 5.76 to 7.61 $\log_{10}$ cfu/ml (1.85 log increase) and from 6.21 to 7.62 $\log_{10}$ cfu/ml (1.41 log increase) as indicated by VPC and FMA, respectively (Fig 4.3). The continuous increase in the number of viable bacteria for encapsulated samples over storage period could be attributed to the ability of the encapsulating matrix to protect the cells from yoghurt acidity. Our results also demonstrate that the encapsulated cells remained well above 6 $\log_{10}$ cfu/ml at the end of the 6 weeks, with counts higher for cells encapsulated with Vegetal-inulin (Fig 4.3), possibly because inulin provided extra solids which have been reported to improve probiotic protection (Capela et al., 2006). These results are in agreement with those of Boeni and Pourahmad (2012), who reported that the addition of 2% inulin improved viability of *Lactobacillus casei* and *L. acidophilus* for 3 weeks. These findings are also similar to those of Akalin et al. (2004), who reported higher counts of *B. longum* in yoghurts containing prebiotics for 4 weeks as opposed to the control. In addition to these studies, improved survival of probiotics in yoghurt with the addition of inulin-type prebiotics have been reported by other researchers elsewhere (Shin et al., 2000; Sadek et al., 2004; Özer et al., 2005; Capela et al., 2006; Aryana and McGrew, 2007).

Our study therefore shows that encapsulation using Vegetal-inulin improved survival of *B. longum* LMG 13197 in yoghurt during six weeks of storage. This might be attributed to the fact that the physical retention of cells within the lipid matrix limited exposure to adverse factors and also the metabolites produced by these cells were minimal and not sufficient to affect cell viability. Based on these results, it could further be speculated that prolonged

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refrigeration of yoghurt containing encapsulated bifidobacteria will not negatively affect the texture and taste of yoghurt. However, this remains to be experimentally determined in future studies.

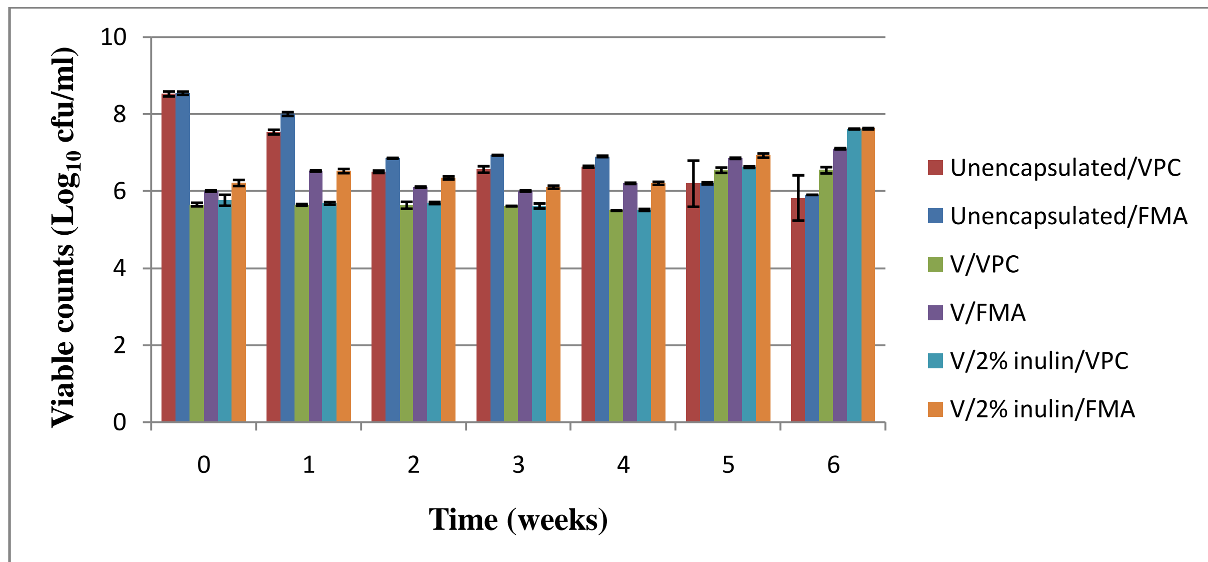

Figure 4.3: Survival of unencapsulated and encapsulated *B. longum* LMG 13197 in yoghurt at 4°C for 6 weeks. V/2% = Vegetal+2% inulin; V= Vegetal; VPC= viable plate count; FMA= fluorochrome microplate assay. Each bar represents the mean of triplicate data from three independent trials; error bars represent standard deviations.

4.4.2 Survival of encapsulated bacteria in yoghurt as determined by flow cytometry

Flow cytometry was used in combination with a Live/Dead Baclight kit L7012 (Molecular Probes) to determine the viability of unencapsulated and encapsulated *B. longum* incorporated into yoghurt. Dot plots of green and red fluorescence were used to differentiate bacterial populations during 6 weeks of storage in yoghurt at 4°C. Each population was represented in quadrants; Q1- injured cells (SYTO9$^+$ PI$^+$); Q$_2$- live cells (SYTO9$^+$ PI$^-$); Q$_3$- unstained viable or lysed cells (SYTO9$^-$ PI$^-$); Q$_4$- dead cells (PI$^+$ SYTO9$^-$). SYTO9 stains all the bacteria cells in a populations, whether dead or alive (Fig 4.4 B and D). PI stains only cells with damaged or compromised bacterial membrane (Fig 4.4 C and E). Dual staining of bacteria cells in a population shows how SYTO9 stains all the cells, but in the presence of PI, SYTO9 is displaced and stains only cells with compromised membranes (4.4 F) (Lahtinen et

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al., 2006). This therefore results in a proper discrimination between live, damaged and dead cells.

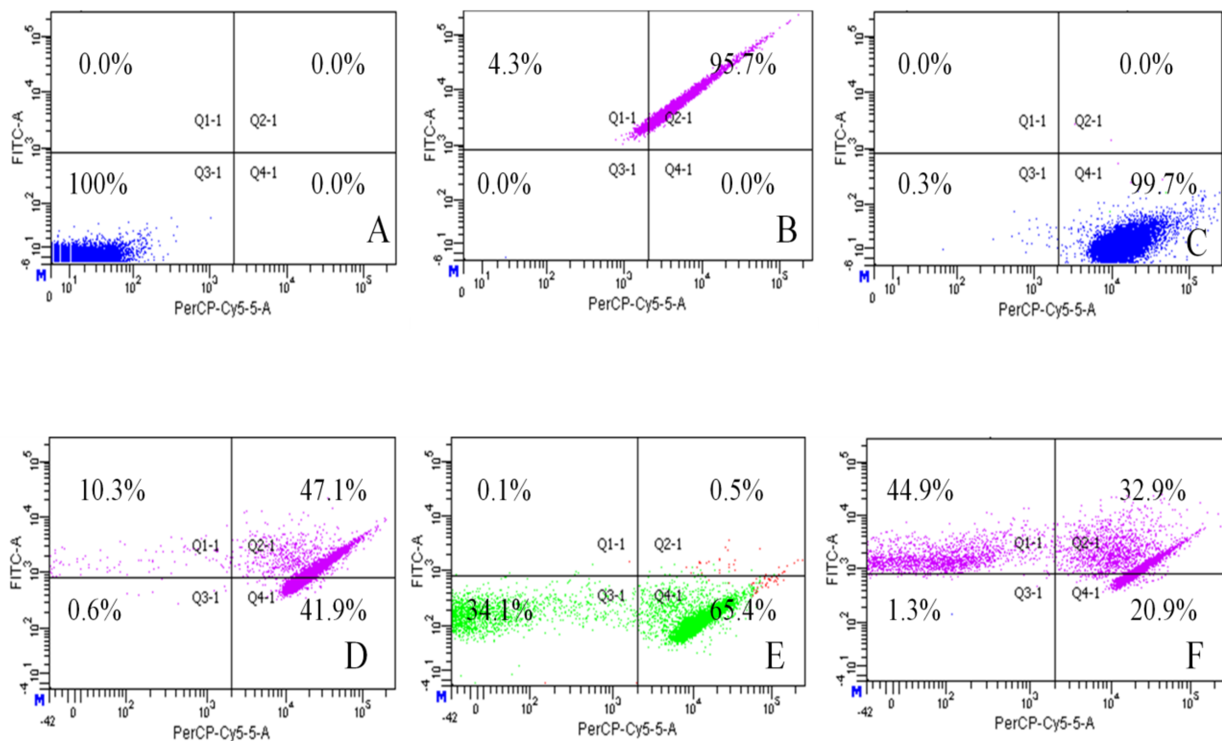

Fig 4.4 Dot plots showing setting of quadrants using *B. longum* LMG 13197 controls: A (Unstained cells), B (SYTO9 stained live cells), C (PI stained dead cells), D (SYTO9 stained mixed cells), E (PI stained mixed cells) and F (Dual stained mixed cells).

Throughout the course of 6 weeks of storage in yoghurt, our results for unencapsulated cells showed that the number of live cells (Q_2) decreased by 37.53%, the injured cells (Q_1) by 5.37% and the dead cells increased by 16.84% (Q_4) (Fig 4.5 A and B). A drastic decrease in the number of viable live cells for unencapsulated cells was apparent after 6 weeks of storage, with a consequent increase in the number of dead cells, suggesting increased cell mortality in the absence of encapsulation (Fig 4.5A and B). There was a good correlation between the percentages obtained from FCA and VPC (Table 4.2), as both methods showed that the number of cells decreased throughout storage period. Light scatter pattern is indicative of bacteria size and granularity or complexity (Veal et al., 2000; Schenk et al., 2011). Light scatter patterns revealed that unencapsulated cells had concentrated to less diffused patterns throughout storage period (Fig 4.5 A and B), suggesting an alteration in the size and internal

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structure of bacteria cells, which is often associated with stress (Hewitt et al., 1999; Cánovas et al., 2007).

After six weeks of storage at 4°C, the population of bacteria encapsulated with Vegetal increased by 15.47% for live cells, with a slight decrease 5.53% for dead cells (Fig 4.5 C and D). On the other hand, the population of bacteria encapsulated with Vegetal-inulin increased by 26.83% for live cells and slightly decreased by 0.57% for dead cells during the same period of storage (Fig 4.5 E and F). An insignificant ($p>0.05$) percentage of injured cells (Q1) and dead cells (Q4) was observed for both encapsulated samples (Fig 4.5 C and D; Fig 4.5 E and F), which suggests that encapsulation in the lipid matrix protected the cells from stressful factors encountered during production to storage. Other researchers have utilised flow cytometry to show improved survival of probiotics after encapsulation (Ananta et al., 2005; Rault et al., 2007; Martin-Dejardin et al., 2013). Since there are limited studies on the shelf life of lipid based synbiotic microparticles encapsulating *B. longum* in yoghurt, no direct comparison to other studies could be made. Results showed that by the sixth week of storage, Vegetal-inulin matrix released a higher percentage of live cells than the Vegetal matrix. There was a good correlation between the results obtained by FCA and VPC (Table 4.2). Light scatter pattern for both encapsulated samples demonstrated a change from slightly dispersed patterns (Fig 4.5 C and E) to a concentrated pattern (Fig 4.5D and F). This suggests that although encapsulation protected the cells during freeze drying, the possible exposure to metabolites during storage could lead to changes in the size and granularity/density of bacteria cells.

The high percentage of unstained cells observed in Q_3 (Fig 4.5) could suggest that; 1) The stress associated with freeze drying reduced the ability of cells to take up SYTO9, 2) The bacterial membrane integrity was relatively high and PI could not penetrate across membrane and stain nucleic acids, 3)The presence of debris which are often seen in treated samples, 4) The presence of yoghurt matrices which could also contain attached cells and 5) The presence of lysed bacteria that may have lost their nucleic acids and therefore cannot be stained (Hayouni et al., 2008; Martinez-Abad et al., 2012). Overall, our results demonstrate that encapsulation with Vegetal-inulin improved survival of *B. longum* LMG 13197 at 4°C after six weeks of storage in yoghurt.

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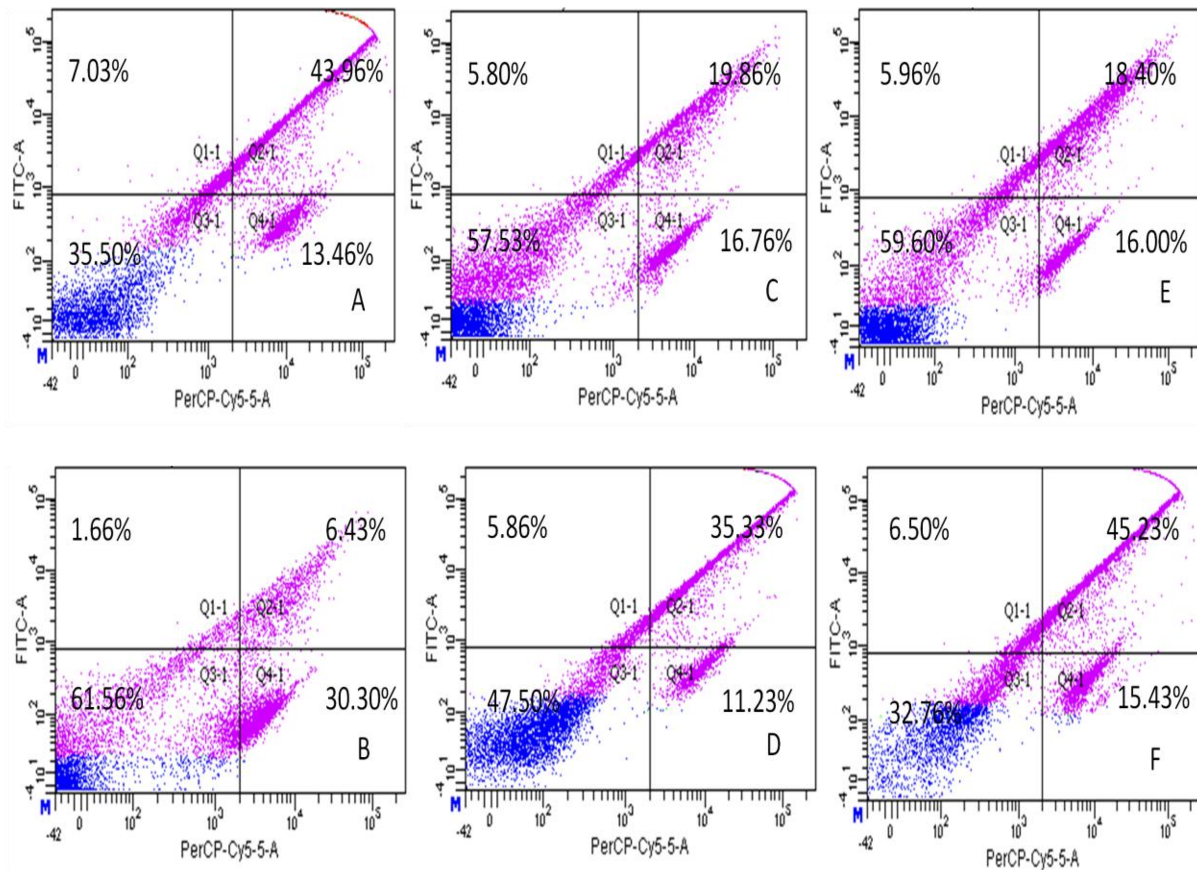

Fig 4.5 Dot plots for *Bifidobacterium longum* LMG 13197 stored in yoghurt at 4°C for 6 weeks: unencapsulated at 0 week (A), unencapsulated at 6th week (B), encapsulated cells without inulin at 0 week (C), encapsulated cells without inulin at 6th week (D), encapsulated cells with 2% inulin at 0 week (E) and encapsulated cells with 2% inulin at 6th week (F).

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Table 4.2: Live counts of unencapsulated and Vegetal encapsulated *B. longum* cells (with and without inulin) obtained by flow cytometry and viable plate count at zero and sixth week of storage in yoghurt at 4°C.

Yoghurt samples	FCM (% live bacteria)		VPC log cfu/ml (%)	
	Storage time (weeks)			
	0	6	0	6
Containing unencapsulated cells	43.96 ± 1.70	6.43 ± 4.66	8.52 ± 0.06 (62)	5.82 ± 0.58 (42)
Containing cells encapsulated in Vegetal matrix	19.86 ± 4.99	35.33 ± 2.53	5.65 ± 0.04 (40)	6.54 ± 0.08 (51)
Containing cells encapsulated in Vegetal-inulin matrix	18.40 ± 6.48	45.23 ± 2.31	5.75 ± 0.14 (46)	7.61 ± 0.01 (58)

Relative percentages of live cells obtained by flow cytometry, total counts log (cfu/ml) obtained by viable plate count and related percentages are represented. Reported values are means of triplicate readings from three independent trials ± standard deviations.

4.4.3 Physico-chemical analysis

4.4.3.1 Change in pH of yoghurts containing *B. longum* during storage

The presence of starter cultures is needed for fermentation of yoghurt, which in turn leads to the decrease in pH levels. This decrease in pH level inhibits the growth of pathogens and thus, improves the shelf life of the fermented products (Rajapaksha et al., 2013). Therefore, pH is an important quality attribute of yoghurt (Tramer, 1973). However, the major problem associated with storage of yoghurt is post acidification mediated by β-galactosidase, which is present in yoghurt but only active at refrigeration temperatures (Cruz et al., 2009). Studies have reported that bifidobacteria alone produces acetic and lactic acids at proportions of 3:2, which when in excess can lead to undesirable effects in yoghurt (Arai et al., 1996; Cruz et al.,

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2009). Therefore, the use of microencapsulation is necessary to reduce and mask negative effects which might lead to unacceptability of the product.

Overall, our results demonstrate that the length of storage period had a significant effect ($p < 0.05$) on the pH of all the samples (Table 4.3). The pH of yoghurt sample containing the unencapsulated *B. longum* LMG 13197 decreased to 4.3 from an initial value of 4.51 (0.21 decrease), with significant differences ($p<0.05$) only observed from the 4th to 6th week of storage (Table 4.3). The continued production of acids by the unencapsulated bifidobacteria cells possibly contributed to the drop in pH (Samona et al., 1996; Marshall and Tamime, 1997; Vinderola et al., 2002; Kailasapathy, 2006). This significant drop in pH would explain the decrease in the number of viable unencapsulated cells (Fig 4.3). Studies have shown that the final pH of yoghurt can affect the viability of bifidobacteria cells (Shah et al., 1995; Vinderola et al., 2000). Akalin et al. (2004) reported that at a final pH of 4.42, a significant decrease in the number of viable *B. longum* stored at 4°C for 4 weeks was observed. Furthermore, our results (Fig 4.3 and Table 4.3) are in agreement with Lankaputhra and Britz (1996), who reported that pH values at or below 4.3 can reduce viability of bifidobacteria. On the other hand, the pH of yoghurt samples containing Vegetal encapsulated *B. longum* LMG 13197 decreased from an initial value of 4.5 to 4.45 (0.05 decrease), with significant effect ($p<0.05$) observed only between the 4th and 5th week (Table 4.3). Similarly, the pH of yoghurt samples containing Vegetal-inulin encapsulated bacteria decreased from an initial value of 4.52 to 4.46 (0.06 decrease), with significant effect ($p<0.05$) also observed between the 4th and 5th week of storage (Table 4.3). The drop in pH of yoghurt samples containing encapsulated cells was not significant ($p> 0.05$) (Table 4.3), partially explaining why viability of the cells was not affected as shown earlier (Fig 4.3). Studies have reported slight decrease in pH values of yoghurt containing inulin over storage time (Paseephol et al., 2008; Boeni and Pourahmad, 2012). Akalin et al. (2004) also reported a 0.06 decrease in pH of yoghurt samples containing *B. longum* and prebiotics after 4 weeks at 4°C.

In comparison to the yoghurt sample containing unencapsulated cells, these findings suggest that encapsulation offered protection to the bifidobacteria cells, and the permeability and solubility properties of the matrix ensured that the metabolites produced by the cells are kept in the matrix and not released into the product, thus minimizing alterations to pH of yoghurt into which they were incorporated. Kailasapathy (2006) reported that post acidification was slower in yoghurt samples containing encapsulated probiotics as opposed to those containing

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free probiotics after 6 weeks of storage at 4°C. Therefore, our results demonstrate that the pH of yoghurt was not affected by the incorporation of Vegetal encapsulated probiotics. The presence of inulin had no significant effect on the pH of yoghurt.

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Table 4.3: pH changes in yoghurt samples containing unencapsulated and Vegetal encapsulated *B. longum* LMG 13197 (with and without inulin) stored at 4°C for 6 weeks.

Yoghurt samples	**Storage time (weeks)**						
	0	1	2	3	4	5	6
Containing unencapsulated cells	4.51±0.01[c]	4.51±0.02[c]	4.50±0.01[c]	4.50±0.01[c]	4.49±0.01[c]	4.40±0.10[b]	4.30±0.01[a]
Containing cells encapsulated in Vegetal matrix	4.50±0.01[c]	4.50± 0.01[c]	4.50± 0.01[c]	4.49±0.01[bc]	4.48±0.02[b]	4.45±0.01[a]	4.45±0.01[a]
Containing cells encapsulated in Vegetal-inulin matrix	4.52±0.01[e]	4.51±0.01[de]	4.51±0.01[cd]	4.49±0.01[bc]	4.49±0.01[b]	4.46±0.01[a]	4.46±0.01[a]

Reported values are means of triplicate readings from three independent trials. Values with different letters within the same row differ significantly ($p<0.05$).

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4.4.3.2 Change in colour of yoghurts containing *B. longum* during storage

The evaluation of colour in food products determines their acceptance or rejection by the consumers, as it is the first and important attribute perceived (Noh et al., 2013). Colour assessment in food products has been used as an indirect measure of pigment contents, as such ensures that the quality of food is maintained (Pathare et al., 2013). The natural pigments which give colour to food products include carotenoids (orange, yellow and red), chlorophylls (green) and anthocyanins (blue, red) (Barrett et al., 2010). Of all the colour systems used for colour measurements, the CIELAB (Commission Internationale de I' Eclairage's) colour system has been commonly used in food industries due to its ability to define two colour attributes a^* and b^* as well as a psychometric index of lightness (L^*) (Sacks and Francis, 2001; Barrett et al., 2010; Granato and Masson, 2010; Pathare et al., 2013).

There were significant differences ($p< 0.05$) in colour attributes (L^*, a^* and b^*) within each of the yoghurt samples containing unencapsulated and encapsulated cells throughout 6 weeks of storage (Table 4.4). The yoghurt sample containing unencapsulated *B. longum* LMG 13197 cells had final values of 84.88, -3.12 and 7.37 for L^*, a^* and b^* attributes, respectively (Table 4.4). On the other hand, the final values obtained for yoghurt containing Vegetal encapsulated cells were 84.51, -3.11 and 7.42 for L^*, a^* and b^* attributes, respectively (Table 4.4). Consequently, the final values obtained for yoghurt containing Vegetal-inulin encapsulated cells were 87.21, -3.14 and 8.61 for L^*, a^* and b^* attributes, respectively (Table 4.4).

With respect to the $L^*$and b^* attributes, results showed an increase in L^* and b^* values with significant differences ($p<0.05$) observed within the samples throughout 6 weeks of storage (Table 4.4). The increase in L^* and b^* values of the samples indicates the tendency towards white and yellow colour. This might be attributed to the colour of the ingredients present in the yoghurt samples which included the whitish colour of inulin and the white to cream colour of milk. The whiteness of milk has been linked to the dispersion of casein micelles and fat globules, which are responsible for the diffusion of incident light (Nozière et al., 2006; Aryana and Mcgrew, 2007). Conversely, studies have stated that yellowness can be linked to the presence of carotenoids in milk (Nozière et al., 2006; Kalač, 2012). Our results

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demonstrate that the presence of inulin had a significant effect ($p<0.05$) on the lightness (L^*) and yellowness (b^*) of yoghurt by the 6th week of storage (Table 4.4). This result is similar to that of Damian (2013) who also reported a high L^* value for yoghurt samples containing inulin.

With respect to the a^* attribute, results showed a decrease in a^* values with significant differences ($p<0.05$) within the samples throughout 6 weeks of storage (Table 4.4). The decrease in a^* values might be attributed to the presence of riboflavin in milk. Riboflavin is a green compound which has been regarded as part of milk’s natural pigment concentration, and thus contributes to the colour of milk (Nozière et al., 2006). Although all the yoghurt samples appeared yellowish-white (cream) in colour at the end of storage period, the total colour difference (ΔE^*) observed between the yoghurt samples containing unencapsulated and the Vegetal and Vegetal-inulin encapsulated cells by the 6th week were 0.37 and 2.97, respectively (Table 4.4). This suggests that the colour changes of the yoghurt samples containing the encapsulated cells are minor to not obvious to the human eye (Martinez-Cervera et al., 2011). Therefore, it could be speculated that this would have no negative effect on consumer acceptability of yoghurt containing Vegetal encapsulated probiotics.

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Table 4.4: Changes in the colour of yoghurt samples containing *B. longum* LMG 13197 cells during storage at 4°C for 6 weeks.

Storage time (weeks)	**Yoghurt samples**											
	Containing unencapsulated cells				Containing cells encapsulated in Vegetal matrix				Containing cells encapsulated in Vegetal-inulin matrix			
	Colour attributes											
	L*	**a***	**b***	**ΔE***	**L***	**a***	**b***	**ΔE***	**L***	**a***	**b***	**ΔE***
0	65.82 ± 0.14^{a}	–1.68 ± 1.10^{c}	–0.44 ± 0.02^{a}	-	63.75 ± 2.01^{a}	–1.30 ± 0.03^{d}	–1.02 ± 0.36^{a}		77.96 ± 1.68^{b}	–2.04 ± 0.18^{c}	3.04 ± 0.95^{b}	
1	78.91 ± 0.51^{b}	–2.19 ± 0.02^{bc}	3.73 ± 0.24^{b}		78.93 ± 1.13^{b}	–2.28 ± 0.18^{c}	4.02 ± 0.77^{b}		68.74 ± 1.06^{a}	–1.11 ± 0.11^{d}	–0.18 ± 0.20^{a}	
2	81.54 ± 0.90^{c}	–2.42 ± 0.12^{ab}	5.63 ± 0.64^{cd}		80.14 ± 2.16^{b}	–2.59 ± 0.31^{b}	4.31 ± 0.65^{b}		78.30 ±0.96^{b}	–2.13 ± 0.17^{c}	3.31 ± 0.46^{b}	
3	82.46 ± 0.24^{d}	–2.81 ± 0.06^{ab}	5.91 ± 0.18^{d}		86.47 ± 0.01^{d}	–3.11 ± 0.08^{a}	8.63 ± 0.07^{e}		86.20 ± 0.13^{d}	–3.18 ± 0.04^{a}	8.22 ± 0.13^{d}	
4	82.20 ± 0.24^{cd}	–2.24 ± 0.03^{bc}	5.37 ± 0.20^{c}		83.77 ± 0.09^{c}	–2.42 ±0.03^{bc}	6.48 ± 0.10^{c}		84.18 ± 0.32^{c}	–2.43 ± 0.03^{b}	6.43 ± 0.13^{c}	
5	85.14 ± 0.26^{e}	–3.04 ± 0.02^{a}	8.33 ± 0.01^{f}		83.19 ± 0.26^{c}	–3.14 ± 0.13^{a}	6.95 ± 0.37^{cd}		86.22 ± 0.10^{d}	–3.20 ± 0.06^{a}	8.82 ± 0.10^{d}	
6	84.51 ± 0.15^{e}	–3.12 ± 0.04^{a}	7.37 ± 0.14^{e}		84.88 ± 0.05^{cd}	–3.11 ± 0.08^{a}	7.42 ± 0.17^{d}	0.37	87.21 ± 0.08^{d}	–3.14 ± 0.01^{a}	8.61 ± 0.10^{d}	2.97

Reported values are means of triplicate readings from three independent trials. Values with different letters within the same column differ significantly ($p<0.05$).

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4.5 Conclusion

This study showed that Vegetal BM 297 ATO is suitable for the encapsulation and protection of *B. longum* LMG 13197 in yoghurt. The presence of inulin improved the survival of probiotics in yoghurt, as bacterial viable counts remained above the recommended therapeutic minimum at the end of the storage period. Post acidification was reduced in the presence of Vegetal-inulin microparticles did not significantly decrease the pH in yoghurt. This suggests that the metabolites produced by the probiotic cells are kept within the matrix; as a result, the taste of yoghurt will not be negatively affected. Colour analysis suggests that the incorporation of Vegetal-inulin microparticles had an effect on the colour of yoghurt, although the total colour change was considered minor or not noticeable to the human eye. Therefore, yoghurt is a suitable vehicle for the delivery of Vegetal-inulin encapsulated probiotics.

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CHAPTER 5

Conclusions and Recommendations

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5.1 Conclusions

- The production of synbiotic lipid based microparticles using Vegetal BM 297 ATO-inulin for the encapsulation of *B. longum* LMG 13197 by freeze drying was successful. High bacteria viability was obtained with non centrifuged formulations containing 2 % (w/v) inulin.

- The Vegetal BM 297 ATO-inulin microparticles gave a high encapsulation efficiency of 82%, which indicates that freeze drying of the *B. longum* within this matrix protected the cells from the detrimental processing conditions associated with freeze drying process, thereby retaining high numbers of viable cells within the microparticles.

- Scanning electron microscopy revealed irregular shaped microparticles, which exhibited rough surfaces and has an acceptable size of 33.4 µm. This indicates that the microparticles will potentially not affect the texture and mouthfeel of the food products into which they will be incorporated. It also showed absence of bacteria cells on the sides or surfaces of the microparticles, as well as an even distribution of the cells within the microparticles, indicting efficient encapsulation.

- Confocal scanning laser microscopy also showed that microparticles contained a high loading of bifidobacteria cells, which suggests that high number of viable probiotics will be delivered.

- Addition of inulin at 2% (w/v) improved the protection efficiency of Vegetal BM 297 ATO. Vegetal BM 297 ATO-inulin matrix protected *B. longum* LMG 13197 in the simulated gastric fluid and displayed significant release of cells in simulated intestinal fluid. More viable cells were present in Vegetal BM 297 ATO-inulin matrix at the end of gastrointestinal transit; therefore this matrix had the potential to continuously release cells into the gut for colonization.

- The recommended level required by food industries for probiotic functions is 10^6 cfu/g. After 6 weeks of storage at 25°C , microencapsulation in Vegetal BM 297

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ATO-inulin matrix by freeze drying improved survival of *B. longum* LMG 13197 by 3 weeks, when compared to the unencapsulated cells.

- Compared to the unencapsulated cells, encapsulation with Vegetal BM 297 ATO did not increase shelf life of *B. longum* LMG 13197 at 4°C. With the addition of inulin, the numbers of viable cells dropped below the recommended levels (10^6 cfu/g) after 5 weeks. This suggests that Vegetal BM 297 ATO-inulin encapsulated bacteria should be stored under refrigeration conditions in order to attain maximum shelf life.

- Vegetal BM 297 ATO is suitable for the encapsulation and protection of *B. longum* LMG 13197 in yoghurt. Viable counts of bacteria remained above the recommended therapeutic minimum after 6 weeks of storage at 4°C. Therefore, encapsulation with Vegetal BM 297 ATO-inulin improved the survival of probiotics in yoghurt.

- There was no significant decrease in pH of yoghurt sample containing Vegetal BM 297 ATO-inulin encapsulated *B. longum* LMG 13197. This suggests that the metabolites produced by the probiotic cells were kept within the matrix, thus the taste of yoghurt will not be negatively affected.

- Although the incorporation of Vegetal-inulin microparticles had a significant effect on the colour of yoghurt, the change was considered minor to the human eye. Therefore, yoghurt is a suitable vehicle for the delivery of Vegetal BM 297 ATO-inulin encapsulated probiotics.

- Based on the above conclusions, synbiotic Vegetal BM 297 ATO-inulin microparticles produced by freeze drying have the potential for application in food industries.

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5.2 Recommendations for Future work

- The use of other prebiotics and lipid matrices for the prolonged shelf life of probiotics should be investigated.

- The effect of other parameters such as oxygen and water activity on the survival of vegetal-inulin encapsulated *B. longum* during storage needs to be analyzed. This will ensure a well rounded understanding of the conditions under which probiotics must be stored.

- Incorporation of other protective agents such as vitamins, plant and fruit extracts alongside Vegetal BM 297 ATO to determine whether improved survival of probiotics can be achieved. This will allow for the production of a variety of formulations using this lipid matrix for improved quality of final products, which will in turn provide multiple essential health benefits.

- Although yoghurt is the most popular and most consumed fermented dairy product, consumers are beginning to show interest in other fermented food products like kefir and mageu. In order to increase the range of food products to which probiotics can be incorporated into, future studies should test the survival of encapsulated probiotics in other fermented products, as well as the effect of their presence on the organoleptic properties of those products.

- Since tolerance of bifidobacteria to detrimental conditions is species as well as strain specific, a wider range of strains from the species tested in the current study, as well as strains from other species, should be encapsulated using the method described in this study and analyze their survival in simulated gastrointestinal fluids and in dairy food products.

- Furthermore, flow cytometry analyses of probiotics encapsulated in the various formulations of Vegetal BM 297 ATO lipid-based microparticles should also take advantage of the cell sorting ability of this technique, by collecting cells that are damaged during exposure to simulated gastric conditions as well as during storage,

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and then performing reinoculation studies. This will go a long way to help confirm possible recovery and regrowth of damaged cells when they are subsequently cultured in a suitable media, or upon their arrival in the colonization site, the colon.

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