Vegetal BM 297 ATO as a potential food grade coating material for microencapsulation of *Bifidobacterium lactis* Bb12 probiotic strain under supercritical conditions

BY

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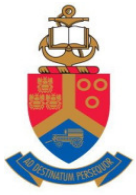

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Declaration

I declare that the thesis entitled "**Vegetal BM 297 ATO as a potential food grade coating material for microencapsulation of *Bifidobacterium lactis* Bb12 probiotic strain under supercritical conditions**", which I hereby submit for the degree of Magister Scientiae at the University of Pretoria, is my own work and has not been previously submitted by me for a qualification at this or any other tertiary institution.

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LIST OF ABBREVIATIONS

AAD: Antibiotic-Associated Diarrhea

CFU: Colony Forming Unit

CSIR: Council for Scientific and Industrial Research

DFA: Direct Fluorescent Antibody

DGGE: Denaturing Gradient Gel Electrophoresis

DNA: Deoxribonucleic Acid

DSC: Differential Scanning Calorimetry

EE: Encapsulation efficiency

ELISA: Enzyme-Linked Immunosorbent Assay

FAO: Food and Agricultural Orginization

FDA: Food and Drug Administration

FISH: Fluorescent In-Situ Hybridization

GBH: Glyceryl Behenate

GIT: Gastrointestinal Tract

GMS: Glyceryl Mono-Stearate

HDL: High Density Lipoprotein

HLB: Hydrophillic-Lipophillic Balance

HPMC: Hydroxypropyl Methylcellulose

LAB: Lactic Acid Bacteria

LDL: Low-Density Lipoprotein

MRS: De Man, Rogosa and Sharpe Agar

NNCP: Nalidixic Acid Neomycin-sulphate Lithium-chloride Paromycin-sulphate agar

PB: Prussian Blue Agar

P_c: Critical Pressure

PCR: Polymerase Chain Reaction

PCR-RFLP: Polymerase Chain Reaction Restriction Fragment Length Polymorphism

PEG: Polythene Glycol

PGSS: Particles from Gas Saturated Solutions

RCPB: Reinforced Clostridial Prussian Blue Agar

rRNA: Ribosomal Ribonucleic Acid

RT-PCR: Reverse-Transcriptase PCR

$scCO_2$: Supercritical Carbon Dioxide

SLP: Solid Lipid Nanoparticle

SIF: Simulated Intestinal Fluid

SGF: Simulated Gastric Fluid

T_c: Critical Temperature

TGGE: Temperature Gradient Gel Electrophoresis

TPPY: Tryptose Proteose Peptone Yeast Extract

TPY: Trypticase-Phytone Yeast Extract

TSA: Tryptose-Soy Agar

UHT: Ultra-High Temperature

WHO: World Health Organization

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SUMMARY

Vegetal BM 297 ATO as a potential foodgrade coating material for microencapsulation of *Bifidobacterium lactis* Bb12 probiotic strain under supercritical conditions

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The use of probiotics administered either in a direct clinical sense or indirectly as a food additive has grown greatly in recent years due to the new 'healthy living' trend. The global market for probiotics was worth R186.2 billion in 2013, showing a growth of 4.3% compounded per annum over a period of five years. Probiotics are however sensitive to different environmental factors such as light, moisture and oxidation in addition to the stresses encountered during processing. The shelf of probiotics in foodstuffs and pharmaceuticals are limited, which lead to increased costs to the manufacturer in having to incorporate enough cultures to account for losses, or a decreased benefit to the consumer in not receiving the required amount of cells needed for health benefits to be actualized. Microencapsulation is a technique used to protect probiotic cells against harsh conditions encountered both in the environment during storage and transport, and in the human body after consumption. This leads to higher numbers of viable probiotic cells being available to the consumer after consumption and decreased costs to the manufacturer as less cells need to be added.

Current microencapsulation techniques are plagued with problems such as the inability to be produced at an industrial scale, the use of toxic solvents which kill the probiotic cells and contribute to pollution and the need for FDA approval of the pharmaceutical grade excipients they use.

A novel encapsulation technique using the Particles from a Gas Saturated Solution (PGSS) system has been developed by the Council for Scientific and Industrial Research (CSIR), which does not require organic solvents and uses carbon dioxide in a supercritical state to create microcapsules.

There is very limited knowledge on the use of lipid based food grade excipients for use in probiotic microencapsulation for food applications. The combination of these ideas could result in a novel method of protecting sensitive food additives, in addition to creating a platform for new techniques in the pharmaceutical and food industries.

Bifidobacteria were successfully encapsulated using the novel PGSS method. Two lipid based excipients, Compritol E 472 ATO and Vegetal BM 297 ATO were tested to determine if they could be used in the microencapsulation of bifidobacteria. Results showed that the temperature needed to successfully liquefy the Compritol resulted in high losses of cells. It was decided to continue the study with only Vegetal BM 297 ATO which resulted in much lower losses of probiotic cells. The results demonstrated that the cells were successfully encapsulated inside Vegetal BM 297 ATO microparticles. The particles contained high numbers of cells and the process did not cause any morphological changes on the probiotic cells. The process was optimized by changing the reaction formulation and mixing chamber parameters until an encapsulation efficiency (EE) of 88% was attained. The Vegetal microparticles containing the bifidobacteria were a very desirable size for use in the food industry. This was the first time the use of a lipid based food grade excipient in microencapsulation of probiotics using a PGSS procedure was demonstrated. This result leads to further testing of the Vegetal BM 297 ATO microparticles containing bifidobacteria in *in vitro* gastrointestinal environments.

It was found that the Vegetal BM 297 ATO matrix provided a protective effect on the cells during simulated gastrointestinal transit. There were more viable cultures in the sample containing encapsulated cells after exposure to simulated gastric fluid (SGF) and subsequently simulated intestinal fluid (SIF). The cells were released from the Vegetal BM 297 ATO matrix over 7 hours after an initial decrease in numbers. The viable numbers of non-encapsulated cells continuously decreased while the encapsulated cells continuously increased over time during exposure.

The microparticles were subsequently tested to see if they increased the shelf life of bifidobacteria under different storage conditions. It was found that during refrigerated storage the microparticles did not increase the shelf life of bifidobacteria, but that it had a slight protective effect when stored at room temperature.

INTRODUCTION

The human gastrointestinal tract plays host to many cells, more so microorganisms than human (Fasoli *et al*., 2003). The human gut microbiome is very diverse in terms of the sheer number of organisms, about 10^{13} , but also the diversity of species, about 500 (Gibson and MacFarlane, 1994). The balance between probiotic and pathogenic gastrointestinal bacteria play a lifelong role in host health (Cummings and MacFarlane, 2007). Good gut health is important in terms of resistance to diseases especially in impoverished areas where it prevents many conditions that require expensive and often dangerous treatments. Maintaining good gut health by consuming a healthy and diverse diet can prevent illnesses which will save governments substantial amounts of resources which would otherwise be allocated to healthcare needed to treat avoidable problems.

Probiotic microorganisms inhabiting the human gastrointestinal tract offer a range of benefits which include but are not limit to detoxification of gut metabolites, increases in non-specific immune response, aid in carbohydrate digestion, deactivation of procarcinogenic compounds and the synthesis of vitamins (O'Sullivan *et al*., 1992; MacFarlane and Gibson, 1997; Rowland and Gangolli, 1999). On the other hand, pathogenic bacteria lead to disturbances collectively termed dysbiosis, which include irritable bowel syndrome, digestive disturbances, diarrhea, production of carcinogens, alterations in endocrine system functioning, hypervigilant immune responses, weight gain due to changes in carbohydrate and fat metabolism, hypercholesterolaemia, fatigue, electrolyte imbalances, atopic dermatitis, depression, anxiety, allergies and metabolic syndrome (Sanders, 1999; Adams and Moss, 2000; Mobelli and Gismondo, 2000; Steyn, 2005; Salminen *et al*., 2010). The pathogens that cause these disturbances are believed to be curbed by probiotics either by inhibition, competitive exclusion or by some inhibitory factor (Sazawal *et al.*, 2006; Doron *et al.,* 2008)

It has been documented that individuals consuming probiotic-rich foods live longer and have better overall health (Mayer, 1948). Longevity as a result of the consumption of fermented milk has been recorded in the Bible and ancient Hindu texts throughout history (Hosona, 1992; Shortt, 1999). The idea of probiotics in terms of health-promoting live organism was first noted by Russian Nobel Laureate, Eli Metchnikoff in 1908. He noticed a sub-population of Belgian

farmers who had a very long lifespan and concluded after studying them for an extensive time period that the drinking of fermented milk and consumption of yoghurt were the cause. He hypothesized that good gut bacteria could suppress or remove what he called the 'putrefactive' or bad bacteria (Mayer, 1948). Although the consumption of sour milk has been practiced in Germany for centuries, Metchnikoff's research was largely ignored by the western world till the late 20th century (Mayer, 1948). The country of Japan adopted the practice in the early 1980's (Ishibashi and Shimamura, 1993) after which it spread to Europe and now enjoys global usage (Tamime *et al.,* 2005). The most widely used application of probiotics is as a food additive to give the food therapeutic quality, thereby turning the food into a medicine. The range of proven benefits associated with the use of probiotics include lowered blood pressure, lowered cholesterol, improvement of immune functioning, aided digestion of fiber and thereby stimulation of bowel movement and neutralization of carcinogenic compounds in the gut (Gilliland and Walker, 1990; Brady *et al.,* 2000; Reid, 2006; Hickson *et al.*, 2007). In order to exert these beneficial effects these organisms need to colonize the epithelium by attaching to the mucous membrane of the gut wall (Salminen *et al*., 2008). If adhesion is not achieved, the organisms would simply pass through the gastrointestinal tract.Current research is focused on the different health benefits associated with these organisms as well as basic physiology and characterization of the different species. Probiotic research also focuses on elucidation of their possible uses in improvement of overall health, industrial processes and food safety (Reid *et al*., 2003).

An organism has a list of prerequisites to adhere to before it can be considered as probiotic, namely; They must be viable and able to reproduce in the human GIT, no adverse reaction to the presence of the organisms should be observed, no adverse reaction due to its metabolic products should be observed, the bacterium should exert an antagonistic effect on pathogens, the bacterium should neutralize carcinogenic compounds and inhibit the pathways that cause them and the organism must be genetically stable and have no mobility genes or genetic system to transfer the gene's (Havenaar and Huis in't Veld, 1992; Lee and Salminen, 1995, Brady *et al.,* 2000).

Probiotic effects are in part due to metabolic byproducts such as hydrogen peroxide, bacteriocins like acidophilic reductions in luminal pH through the production of volatile fatty

acids and even regulation of the colonic cells themselves through regulation of the gene responsible for the production of mucin which impedes adherence (Mack *et al.*, 1999). Independent studies have however shown that bifidobacterial spp. are cleared form the GIT within 8 days of ceasing intake which shows that continuous administration is needed. The organisms need to be taken in at a minimum of 10^9-10^{10} cfu per day to exert probiotic effects on the host (Fasoli *et al.*, 2003).

Probiotic cultures have short shelf lives in refrigerated products due to unfavorable conditions and low viability over time in dried products (Chen and Yao, 2002). This is attributed to stressors such as processing, light, moisture and oxygen. In addition to these outside factors they are subjected to unfavourable conditions in the human body such as the low pH of the stomach (<2.5) and the presence of bile salts, which result in a loss of viable cells (Heidebach *et al*., 2012). The result is a decrease in the number of viable cells that transit to the small intestine and colon where adhesion of these cells needs to occur. If there are fewer cells present at the adhesion site the chance of successful adhesion and subsequent colonization decreases, which needs to occur for health benefits to be realized (Mack *et al.*, 1999). If the cells could be protected against these harsh environments and reach the adhesion sites in higher numbers, fewer cells would have to be added to the initial foodstuff to account for losses, which would result in a significant cost saving to the consumer.

Microencapsulation is a technique that involves entrapping microbial cells or bioactive compounds in a semi-permeable, either organic or inorganic, polymeric gel structure in order to protect it from some factors that might decrease the efficacy or viability of its contents such as light, moisture, oxidation and acids (Kim *et al.*, 2008). This allows for the targeted delivery of microorganisms as well as increasing of shelf life thereby yielding more viable cultures to colonise the gut. The current methods used in microencapsulation are plagued with difficulties such as use of toxic organic solvents, high temperatures, low production capacity, large microcapsule diameters and low encapsulation efficiency (Heidebach *et al*., 2012). The different coating materials must meet FDA standards before it may be used as a coating material and even then many of the polysaccharides are not permitted in certain foodstuffs (Picot and Lacroix, 2004). The pharmaceutical nature of the polymers therefore presents problems.

A shift needs to be made toward food grade coating materials which are generally regarded as safe (GRAS) and do not need FDA approval, together with the use of novel encapsulation methods.

The use of supercritical fluids to create particles from a gas saturated solutions for the microencapsulation of probiotics has recently been investigated and shown to have great promise (Moolman *et al.*, 2006). It allows for the use of carbon dioxide which is a stable, inexpensive, non-toxic gas, thereby negating the use of solvents or surfactants, it produces microcapsules of a desirable diameter for use in food applications and does not harm sensitive bioactives like probiotic cells.

There has been a recent increase in the public interest in probiotic cultures and the benefits they offer. Probiotic cultures are already common additions to many products used daily in most households and already have a strong market for themselves in the pharmaceutical industry with a reported market value of $19.6 billion in 2013 (BioGaia, 2013). More research needs to be done to assess the suitability of food grade excipients for use in the encapsulation of probiotics due to a current lack of knowledge on the subject. This research should be done in order to expand the current market for probiotics and improving current techniques used in targeted-delivery systems.

The main aim of this study was to assess the suitability of Compritol E 472 ATO and Vegetal BM 297 ATO as food grade coating materials for the encapsulation of bifidobacteria using a PGSS method with supercritical carbon dioxide as a solvent.

The specific objectives were to successfully encapsulate *B. lactis* Bb12 in both materials and to determine the characteristics of the resulting microparticles. The particles were then tested for stability during simulated gastrointestinal transit and shelf life studies.

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CHAPTER 1

LITERATURE REVIEW

1.1. MICROFLORA OF THE GASTROINTESTINAL TRACT

The gut is colonized at birth by a succession of different microorganisms, the balance of which has an intimate role in the health of the host throughout his/her life. The balance between beneficial and detrimental bacteria play a major role in overall health of the gut and therefore the individual. The human gut is very diverse in terms of the amount of organisms, about 10^{13} , but also the diversity of species, about 500 as of yet (MacFarlane and Gibson, 1997; O'Riordan *et al.*, 2001).

It is very important to maintain good gut health especially in communities where a healthy and balanced diet is not always the option. This is usually the result of isolation from reliable food sources and lack of income or knowledge concerning the subject. Maintaining good gut health and by consuming a healthy and diverse diet can save individuals as well as governments substantial amounts of resources spent on the healthcare needed to treat avoidable problems (MacFarlane and Gibson, 1997). Basic research into food science in all its forms is needed as it is a field where a massive impact can be made on the health and quality of life of countries' citizens.

The probiotic microorganisms inhabiting the human gastrointestinal tract offer a range of benefits which include but are not limited to the detoxification of gut metabolites, increases in non-specific immune response, aid in carbohydrate digestion, deactivation of procarcinogenic compounds and the synthesis of vitamins (O'Sullivan *et al*., 1992; MacFarlane and Gibson, 1997, Rowland and Gangolli, 1999).

The well adapted nature of these autochtonous inhabitants of the gut make colonization by other microorganisms very difficult, this phenomenon is known in the literature as non-specific disease resistance that is an important hurdle in protection of the gut. The monogastric GIT can be accurately re-created *in vitro* for the running of simulations.

The gut microflora have a direct effect on many factors pertaining to gut metabolism such as short-chain fatty acid production, enzymatic activity in intestines, redox potential of luminal contents, host physiology, host immunology and modification of host-synthesized molecules (Tannock, 1995). Among the short chain fatty acids produced by these organisms in the lumen

acetate butyrate and propionate are the most produced. These may contribute as much as 10% of the hosts daily energy requirements as most of the short-chain fatty acids are absorbed through the gut wall (MacFarlane and Gibson, 1997). The epithelial cells obtain two thirds of their energy from butyrate (O'Sullivan, 1996). The short chain fatty acids also help with control of luminal pH, mineral ion absorption and the regulation of metabolic pathways.

A disturbance of the gut microbiota, such as the administration of an antibiotic, kills most of the non-resistant bacteria in the gut. This may eliminate a stable population of probiotic organisms adhering to the gut wall, giving enteropathogens like *Clostridium difficile*, the organism linked to pseudomembranous colitis, a chance to outcompete and irritate the bowel, causing conditions such as gastroenteritis and diarrhea. The human GIT is thus in a constant struggle for homeostasis with respect to the relative abundances of the different species living there. Other harmful organisms that have been linked to certain chronic conditions are *Escherichia coli*, which harmful effects are usually held at bay by the normal gut population but can exert its pathogenic effects when this population is disturbed. It has been linked to a condition called ulcerative colitis along with *Streptococcus mobilis*, fusobacteria and shigella. Sulphate reducing bacteria are also implicated (MacFarlane and Gibson, 1997).

The GIT is an essential organ system and a disruption in normal gut functioning can cause great discomfort or even a life threatening situation in cases such as necrotizing enterocolitis, gastroenteritis or gastro/enterosacromas (Salminen *et al.*, 1988). It is inhabited almost exclusively by anaerobes such as bacteroides, eubacteria, bifidobacteria, clostridia, lactobacilli, ruminococci, and streptococci.. The native gut microflora are extremely well adapted to their environment but their interactions with the local and expanded areas of the body are still poorly understood (MacFarlane and Gibson, 1997).

1.1. PROBIOTICS

Probiotics are described by the FAO/WHO as 'live organisms which when administered in adequate amounts confer a health benefit on the host' (FAO, 2004). The two genera to which most probiotic species belong are bifidobacterium and lactobacillus, with the recent addition of leuconostoc and pediococcus.

Current research is focused on the different health benefits associated with these organisms as well as basic physiology and characterization of the different species. Probiotic research also focuses on elucidation of their possible uses in improvement of overall health, industrial processes and food safety (Reid *et al*., 2003). There has been a recent increase in public interest in probiotic cultures and the benefits they offer. Probiotic cultures are already common additions to many products used daily in most households and already have a strong market for themselves in the pharmaceutical industry with a market value of $19.6 billion in 2012 and predicted to increase to $23.93 billion by 2017 (Yahoo Financial Report, 2013). The range of benefits associated with the use of probiotics such are lowered blood pressure, lowered cholesterol, improvement of immune functioning, aided digestion of fiber and thereby stimulation of bowel movement and neutralization of carcinogenic compounds in the gut (Gilliland and Walker, 1990; Brady *et al.*, 2000; Reid *et al*., 2003; Hickson *et al.*, 2007). They are most commonly used in a clinical capacity to prevent diarrhea due to secondary infection by pathogenic intestinal organisms such as *C. difficile* after a course of antibiotics (McFarland, 2006).

These pathogens are thought to be held at bay by probiotics either by inhibition, competitive exclusion or by some inhibitory factor (Sazawal *et al.*, 2006; Doron *et al.*, 2008). In order to exert these beneficial effects these organisms need to colonize the epithelium by attaching to the mucous membrane of the gut wall (Salminen *et al.*, 1988). If adhesion is not achieved, the organisms would simply pass through the intestines (Lankaputhra and Shah, 1995). It is therefore a crucial first step in the realization of the metabolic cascade ending in beneficial outcomes to the host.Probiotic effects are in part due to metabolic byproducts such as hydrogen peroxide and bacteriocins like acidophilin which result in reductions in luminal pH through the production of volatile fatty acids and regulation of the colonic cells themselves through regulation of the gene responsible for the production of mucin which impedes adherence have been cited (Mack *et al*, 1999).

Many potential pathogens exist within the microbial flora found in the human gut and can exploit any change in the condition of its environment. This may result in a pathogenic organism having the advantage that gives it an opportunity to outcompete the natural microflora and cause an acute inflammatory reaction such as enterocolitis or inflammation of the gut wall (Salminen *et al.*, 1988).

This type of reaction can be made manifest by many genera including aeromonas, campylobacter, clostridium, escherischia, salmonella, shigella and yersinia to name but a few (McFarland, 2006).

An organism has a list of prerequisites to adhere to before it can be considered as probiotic, namely; They must be viable and able to reproduce in the human GIT, no adverse reaction to the presence of the organism should be observed in the host, no adverse reaction due to its metabolic products should be observed, the bacterium should exert an antagonistic effect on pathogens, the bacterium should neutralize carcinogenic compounds and/or inhibit pathways that cause them, the organism must be genetically stable and have no mob genes or genetic system to transfer the gene's (Havenaar and Huis in't Veld, 1992; Lee and Salminen, 1995).

In addition to the many perils an organism will face in the GIT, it must not just survive their but flourish there. Manufacturers of probiotic or neutraceutical products must ensure that the organisms can survive these barriers as dictated by regulatory authorities. Tannock *et al.* (1995) proposed the following theorized roles that the bacterium should fulfill including but not limited to; stabilization of homeostasis among gut microflora ,protection against enteropathogens, reduction of serum cholesterol, reduction of mutagenic enzyme systems in the gut, assist in lactose digestion, improvement of immune response, increase in calcium absorption, synthesis of vitamins and predigestion of proteins.

Academic studies have shown that bifidobacteria are cleared from the GIT within 8 days of ceasing intake. The need for constant intake must thus be overcome as well. The organisms need to be taken in at a minimum of 10^9-10^{10} cfu per day to exert probiotic effects on the host (Fasoli *et al.*, 2003). Probiotic cultures have short shelf lives in refrigerated products due to low temperatures and low viability over time in dried products. They undergo many stresses such as light ,moisture and oxidation. As they are strict anaerobes oxygen stress is a major threat to cell viability (Fasoli *et al.*, 2003).

1.1.1 Functional Foods

While probiotics are mainly delivered in the form of dietary supplements, many are being incorporated into everyday foods to market them as 'functional' foods. Probiotics cultures like

Lactobacillus delbreuckii subsp. bulgaricus and *Streptococcus thermophilus* have been incorporated into juices, candy bars granola, yoghurt and cookies. The amount of viable cultures at time of consumption should be high enough, usually 10^7 cfu/g of product, to be classified as a functional food (Fasoli *et al.*, 2003). A problem that has been encountered is that the standard held for these products does not test each strain respectively, they only have to meet the requirement together.

This leads to the problem that numbers of one strain may be much higher a time of manufacture and therefore in final product. The product should contain the amount of cultures stated on the container until the expiry date is reached (Fasoli *et al.,* 2003). These products are often sold in speciality or health food stores.

Other probiotics are obtained through spontaneous fermentation in the preparation of traditional foods. Two traditional cereal based non-alcoholic fermented products are called amahewu and incwancwa. Amahewu is a sour maize-based fermented gruel/beverage consumed mainly in the rural areas of South-Africa. Maize is the main substrate used in its fermentation. Amahewu (Also known as Mageu) can be kept in a cool place for many days and serves as an energy drink both for adults and children. The preparation involves mixing maize meal to water in a 1:9 ratio and then boiling with occasional stirring for 10-15 minutes. It is then cooled to about 35-40°C and a source of inoculum (usually flour) is added to start the fermentation process. It is then allowed to ferment for 24-72 hours in a warm place (Steyn, 2005).

Incawanca is a different non-dairy based non-alcoholic traditional fermented beverage. The preparation follows in the footstep of amahewu in that maize meal is fermented over a three day period using spontaneous fermentation by cultures found naturally in the maize. The exception is when the process is done, the mix is then cooked, and salt and sugar is added to taste. This will naturally also kill any wanted bacteria rendering the addition of them moot. The main disadvantage of beer is that it is only consumed by 4.2% of the population, severely restricting access to the majority of the population (Steyn, 2005).

1.1.1.1 Applications

It is well documented that probiotics have been actively applied in foods, turning the foodstuffs into so-called 'functional foods' for centuries wall (Salminen *et al.*, 1988). The attribution of long life to the drinking of sour milk string from biblical times but the active application has been practiced in Germany for century (Mayer, 1948). The country of Japan adopted the practice in the early 1980's (Ishibashi, 1993) after which it spread to Europe and now enjoys global usage (Tamime *et al.*, 1995). The most widely used application is yoghurt containing bifidobacterial cultures. It has been shown in a study performed by Kullen *et al.* (1997) that consumption of probiotic yoghurt on a daily basis led to an increase in bifidobacterial species secreted in feces. The rate of increase peaked at 4 days after daily consumption and then declined till feeding stopped at 8 days. At 16 days (8 days after feeding stopped) total washout of the organisms were observed. This suggests that continual consumption of the organisms is necessary for a stable gut population to remain. This is testament to the viability of the cultures in when administered in a food matrix such as yoghurt.

1.1.1.1.1 Antibiotic Associated Diarrhea

The use of probiotics in the prevention of antibiotic associated diarrhea has attracted a lot of research over the years. Antibiotics kill beneficial and detrimental bacteria alike, and so wipe out most of the natural microflora in the GIT. When the organisms start re-growing however, the detrimental organisms grow faster than the beneficial ones. This results in overpopulation by organisms such as *Clostridium difficile* which results in poor carbohydrate metabolism, inflammation of the gut wall and diarrhea (Ouwenhand *et al.*, 2002). When probiotics are concomitantly administered with antibiotics they adhere and colonize the gut faster than the detrimental organisms, thereby preventing diarrhea.

1.1.1.1.2 Antitumorogenic activity

They have been shown to inactivate colonic enzymes such as glucoronidase, azoreductase, glycoholic acid hyrolase (Chen and Yao, 2002) which convert procarcinogens to carcinogens such as nitrosamine which is able to react with DNA molecules. Lower levels of these

carcinogens will translate into a lower risk of cancer of the lower GIT. The main preventative measure against colorectal cancer is associated with the production of short-chain fatty acids. Lyophilized *B. longum* BB536 reduced carcinogenesis induced by an imidazoquinolone or azoxymethane derivative (Riverson and Reddy, 1993). *B. longum* has also been shown to increase bone strength as a result of increased calcium absorption (Igarashi *et al.*, 1994).

1.1.1.1.3 Depression and Anxiety

Probiotics have been shown to mitigate the effects of depression and anxiety, through its effect on L-tryptophan, a precursor of serotonin, a neurotransmitter shown to be involved with feelings of happiness and wellbeing. An increase in bifidobacteria and lactobacilli correlated with a significant decrease in anxiety symptoms, while no such response was seen in the placebo group. Bifidobacteria have been shown to increase the level of tryptophan in the brain of a rodent animal model, so negating the need for medicines which may have dangerous side-effects (Logan et Katzman, 2005; Venket-Rao et Bested, 2012).

1.1.1.1.4 Serum cholesterol and heart disease

Low density lipoproteins (LDL) transport cholesterol from the liver to various tissues throughout the body. High levels of cholesterol in tissues form deposits in arteries leading to conditions such as arthrosclerosis. High density lipoprotiens (HDL) transport cholesterol from the tissues back to the liver for metabolism. Adequate levels of probiotics have been shown to lower the levels of serum cholesterol by compared to a control group. A study by Kiessling *et al.* (2002) showed no significant change in LDL or serum cholesterol levels but an increase of 12 mg/dL in HDL. A concurrent study by Algerholm-Larsen *et al.* (2002) showed by double-blind trials that probiotics administered through a yoghurt vehicle lowered serum cholesterol levels by (8.5 mg/dL) 4% and lowered serum LDL by 5% (7.7 mg/dL).

1.2. BIFIDOBACTERIA

Bifidobacteria are Y or V-shaped although they may also be spatulate or club-shaped (Charteris *et al.*, 1997) and belong to lactic acid bacteria. Their optimum growth temperature is between 37ºC - 43ºC and they perform best at a pH of 6.5 to 7.0 (Roy, 2001).

They do not produce spores and promote good intestinal health in humans and animals due to their health benefits (Ouwehand *et al*., 2002).

It was first isolated in 1899 by Tissier of the Pasteur institute in France from a healthy breast-fed infant. The name signifies the shape, or better morphology of the bacteria. Bifidus means cleaved in two in Latin. In the 1960's the bacterium, once placed in the lactobacillus genes as *Lactobacillus bifidus* was recognised as an independent genus.

The main species isolated from the human colon and are used in probiotic products are *B. adolescentis*, *B. bifidum*, *B. infantis*, *B. lactis*, *B. breve, B. pseudocatenulatum* and *B. longum* (O'Sullivan *et al.,* 1992; Roy, 2001, Ouwehand *et al.*, 2002). The species associated with animal digestive tracts are *B. pseudolongum, B. thermophilum* and *B. animalis* (Ouwehand *et al.*, 2002).

Bifidobacteria produce organic acids by their breakdown of glucose and mothers' milk (Tannock, 1997). They are distinguished from *Lactobacillus* species by the presence of fructose-6-phosphate phosphoketolase enzyme (Doleyres and Lacroix, 2004). The species associated with the human digestive system are never isolated from animals and vice versa. The reason for this specificity is not known, but most likely due to receptor specificity in terms of adhesion.

It has been shown to be able to pass the barriers associated with the infant gastrointestinal tract (Lankaputhra and Shah, 1995; Brady *et al.*, 2000; Ouwenhand *et al.*, 2000) where it adheres to the epithelial cells and proliferates (Charteris *et al.*, 1997). *Bifidobacterium infantis* is one of the first colonizers of the infant gut (Guarner and Magdalena, 2003), where it is thought to play a role in protection against pathogenic species as well as aiding in establishment of a stable, dynamic, adult species distribution of intestinal micro-organisms (Björkstén *et al.*, 2001).

Bifidobacterium sp. constitute approximately 90% of the gut microbiota of infants that are born by natural delivery, where they are received from the mother through ingestion of fluids in the birth canal (Guarner and Magdalena, 2003).

The figure for infants delivered by Cesarean section is significantly lower (Gibson *et al.*, 2000). Infants delivered by caesarean section and therefore did not receive the normal microbiota that a child that passes through the birth canal during natural delivery would (Björkstén *et al.*, 2001). The adult composition is only about 3-6 % (Charteris *et al.*, 2005).

Bifidobacteria which are present in high numbers gradually decrease as the infant is weaned and other genera such as eubacteria and bacteriodes become predominant (Reid, 2006). Bifidobacteria are one of the most important bacteria in adulthood, still being a major component of the gut microflora. The numbers of bifidobacteria decrease with age, while pathogenic species such as clostridium increase (Charteris *et al.*, 2005). The presence of bifidobacteria has been linked to among other beneficial effects which include; improvement of intestinal flora, inhibition of intestinal putrefactive substances, alleviation of constipation and alleviation from diarrhea (Gibson *et al.*, 2000).

They are known to produce acetic and lactic acid which in addition to lowering luminal pH, has a strong antibiotic effect against harmful bacteria (Charteris *et al.*, 2005). Bifidobacteria have a unique fructose-6-phosphate phosphoketolase pathway by which to anaerobicly ferment carbohydrates. Bifidobacteria play a role in improving immunological functions by inducing both specific and non-specific antibody production. This occurs by assimilation of whole cell or cell component by the macrophages. Antitumor effects and non-specific resistance to toxins have also been reported by Yamakazi *et al*, 1991.

One of these probiotic species, *Bifidobacterium bifidum*, a non-motile, gram positive fastidious anaerobe, that is already widely used to prevent antibiotic associated diarrhea and has been demonstrated to inhibit certain pathogenic bacteria, improve immune function, assist in the synthesis of certain vitamins and has not been linked to any clinical symptoms or illnesses in addition to meeting all of the abovementioned criteria (Björkstén *et al.*, 2001).

1.3. MICROENCAPSULATION

Microencapsulation is a technique that involves entrapping microbial cells or bioactive compounds in a semi-permeable either organic or inorganic polymeric gel structure in order to protect it from some factor that might decrease the efficacy or viability of its contents such as light, moisture, oxidation and acids. (Kim *et al.*, 1988). This allows for the targeted delivery of microorganisms as well as increasing of shelf life thereby yielding more viable cultures to colonise the gut. Different coating materials must meet FDA standards before it may be used as a

coat material. The generally regarded as safe (GRAS) classification system governs this. There are different techniques of microencapsulation the two main categories being chemical and physical. There are different methods described by Anal and Singh (2007), some of the most common are;

- Spray-drying
- Freeze-drying
- Spray-coating
- Extrusion
- Emulsion
- Supercritical fluid plasticization
- Supercritical Carbon dioxide

1.3.1 Spray drying

The use of spraying techniques allows for the entrapment of bioactives or food products in liquid form by dehydrating them. This involves atomizing the liquid into droplets in a container and drying the very small (therefore very large surface area in contact with hot air) particles when compared to original product in a stream of hot, dry air. The moisture evaporates upwards with the hot air while the dehydrated powder remains behind. The powder is cooled and packaged according to specifications of product. A careful equilibrium must be taken between having a high enough temperature to sufficiently remove the moisture but not too high as to give rise to heat damage. The typical temperature of the drying state is 45-50°C and so some bacterial suspensions can be safely made without killing the cultures (Fritzen-Freire *et al*, 2012).

The shape and size of the particles are determined by the process of atomization. There are two main principles of atomization used in spray drying, centrifugal force and high pressure spray jets. Centrifugal force involves a spinning disk called a cyclone spray drying system. The disk rotates at between 2000-20000rpm. The bioactive or food is delivered near the centre and is spread out onto the radial plane trough two thin sheets of metal. Surface tension immediately causes the droplets to take on a spherical shape. This technique is preferred for non-homogenous substances as the spinning disks don't clog very easily. The high pressure spray jet principle works by mixing a high pressure stream of liquid with a high pressure stream of gas. The liquid

then exits the nozzle in a swirling motion and forms droplets in a cone shaped sheet. Drying of the droplet then occurs by evaporation of moisture from the material. The drying time is proportional to the square of the droplet radius. The drying time can be reduced by pre-concentrating the liquid so lowering the initial moisture content (Karel *et al.*, 1975).

The addition of prebiotics has attracted attention such as is demonstrated in the study by Fritzen-Freire *et al* (2012) where *B. lactis* Bb12 was microencapsulated by spray drying with different mixtures of inulin, oligofructose or oligofructose-enriched inulin. These prebiotics replaced reconstituted skim milk as the normal encapsulating agent. The microcapsules containing the prebiotics had higher initial counts of the bacteria in addition to being more thermally stable. The water activity was however lowered but no effect on viability was reported (Fritzen-Freire *et al*, 2012). There is a clear loss of viability due to the atomization step as a result of the temperature changes and dehydration. An advantage of this method is that larger quantities of microcapsules can be produced at low cost because the equipment is readily available as well as affordable (Picot and Lacroix, 2004).

1.3.2 Freeze-Drying

The freeze-drying technique uses a molten matrix with a low melting point. The bioactive compound is then atomized by spraying it trough a nozzle into a vessel containing said matrix. Cold air is then injected into the chamber, solidifying the matrix, this freezing step can take between 1 and 24 hours. The longer the freezing step the bigger the particles (Franks, 1997). The bioactive compound is now encapsulated in the gel matrix the size of which can be adjusted by modifying the temperature and nozzle diameter. The next step is the sublimation step. The particle is then dried, but unlike spray drying this happens with the product under its critical temperature. It works by creating a strong vacuum into which the moisture will evaporate (same effect as boiling), and will be left with a moisture content of just 3-5%. A second drying or 'desorption' step removes any water than is ionically bound from the particle (Franks, 1997). This process is also known as spray chilling. A viable cell load of $10^{10\text{-}11}$ cfu/g powder can be achieved when encapsulating bacteria.

A problem that has been encountered is the relatively short duration of the protective effect (Dolyres *et al.*, 2005). It is stated that other techniques are more suited in the laboratory setting

due to the equipment needed for the process being expensive and not suited for a laboratory setting (Champagne *et al.*, 2005). Freeze-drying has been used by Semyonov *et al* (2009) to produce dry microcapsules consisting of trehalose and maltodextrin containing viable lactobacillus cells. It was compared to bulk freeze drying, and could generate cells with a high viability (above 60%). The main factor was however found to be concentrations of the trehalose during the freezing stage. The technique is therefore effective but in its infancy and needs more refining before large scale ambitions can be actualized.

Spray chilling is a difficult technique to master and scientists in the field of microencapsulation need to focus on creating smaller beads to allow for better application in food products, testing of a larger range of food products and they also need to focus on industrial scale systems for research (Gouin, 2004). Large scale processes are the only ones that will be economically viable creating an income to further basic research.

The industries already implementing their different practices should also release their data to the academic domain to the end of speeding up the processes leading up to successful implementation of ideas in the field.

1.3.3 Spray-coating

Spray coating methods include fluidised bed, Wurster and Tangential technologies. It involves a coating material , usually in liquid form, being sprayed over the core material to solidify and form a layer at the surface. The material is usually atomized and injected from many angles. The number of angles can be modified to influence the characteristics of the coating material. This method is often used for probiotics. The viable load is very high at $>10^{11}$ cfu/g powder (Dolyres *et al.*, 2005). It offers high protection during short periods of exposure to undesirable environments such as those with low pH, high humidity and high concentrations of oxygen (Gouin, 2004). If need be, multiple layers can be added to increase protective measures though this will increase cost and cell release time, especially at low temperatures. There is also a chance of a phase separation occurring in beverages if the coating is lipid based. It is due to its low cost the most used technique in probiotic microencapsulation.

1.3.4 Extrusion

The extrusion is a technique whereby a bacterial culture suspended in alginate or a sodium alginate solution is dropped into an aqueous $CaCl_2$ solution. This causes the alginate beads containing multiple viable bacterial cells to solidify. The alginate beads are then immersed in a chitosan solution which coats the alginate beads in an outer layer of a supportive material (Fig 1.1). The size of the particles can be adjusted using vibrating nozzles or piezzo effects. This is a measuring technique whereby an electrical potential appears across faces of a crystal (most solids) when it is subjected to mechanical pressure. A scale, car throttle or modern thermometer for example. The electrical potential will thus affect the charge of the droplets as they repel or attract each other, this can be controlled by reversing the effect and applying mechanical stress instead of measuring it.

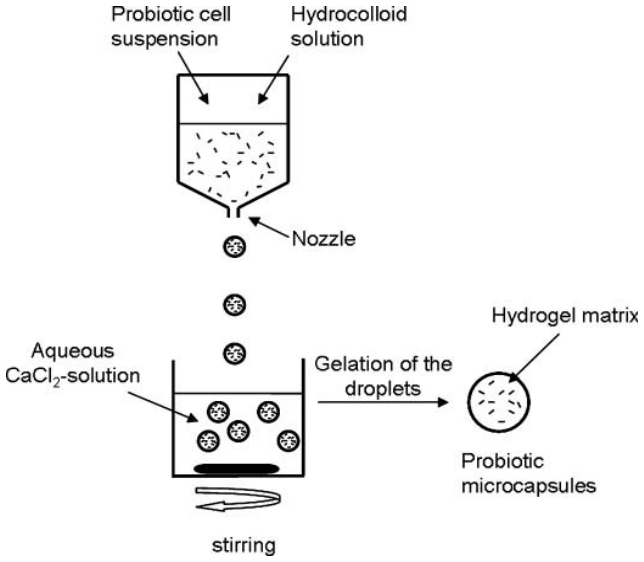

Figure 1.1 Diagram depicting the extrusion process (Heidebach *et al.,* 2012)

1.3.5 Emulsion

The emulsion technique is carried out by adding a k-carrageenan or alginate suspension to an oil phase. The mixture can be solidified through the addition of a $CaCl_2$ (aq) solution, which will provide Ca^{2+}, or by addition an acid. When the ingredient is added to the k-carrageenan, co-encapsulation occurs. The solidified beads containing the ingredient are then exposed to the chitosan solution to obtain an outer layer of coating. In the third technique, also known simply as

the emulsion process $CaCO_3$ (aq), cells and carbonate (CO_3^-) are suspended in an alginate solution. The solution is the dripped into an oil phase creating round beads. The beads are then solidified by adding an acid solution, the carbonate acting as a buffer. Upon solidification the chitosan coating step is done (Fig 1.2). Many different supportive coating materials such as gelatine, cellulose acetate phthalate, microcrystalline cellulose can be combined with the primary coating material. With the two emulsion techniques the particle size is controlled by agitation speed and conception of the mixers. The size is usually 25µm-2000µm. Chandramouli *et al* (2004) demonstrated that the viability of cells trapped in alginate microspheres increases in gastric conditions with an increase in gel concentration and particle size. Wunwisa *et al* (2005) observed a cryprotective component of alginate beads coated with chitosan in normal and UHT-trated milk stored at low temperatures for 4 weeks. The survival of the microencapsulated bacteria was higher by about 1 log cycle and was still above the therapeutic minimum of 10^7 cfu/g. It is however very difficult to produce large quantities of particles.

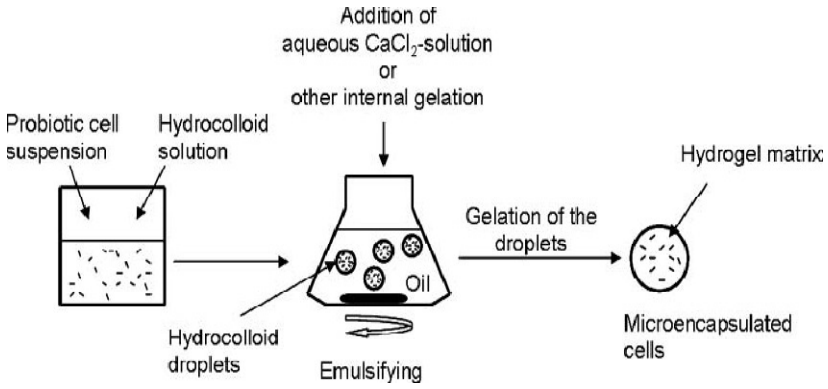

Figure 1.2 Diagram depicting the emulsion process (Heidebach *et al.,* 2012)

1.3.6 Supercritical Fluid plasticization

Supercritical fluids are being looked at as a new revolution in encapsulation techniques as they offer great advantages over the presently used methods. As many fluids are biodegradable and are use to encapsulate or co-precipitate natural products supercritical fluid congealment holds great advantages in fields such as cosmetics, pharmacology and food science. The core material

to be encapsulated is usually covered (or encapsulated) in a thin layer of a non-polar coating substance, usually a biopolymer of fat.

A supercritical fluid is defined as a fluid that is above both its critical temperature and pressure. This state allows properties of the fluid's nature such as density, viscosity and dielectric constant to be 'adjusted' by tuning the temperature or pressure (Cocero *et al.*, 2008).

Supercritical fluids have essentially the density of fluids ranging from 0.1-1g/ml, thereby making them good solvents (Krober et Teipel, 2005). They are however not fluids. The processes that are associated with supercritical fluids are based on the specific properties of the fluids in question.

The rule is that good solvent power is achieved when fluid is near T_C (critical temperature) and much over P_C (critical pressure). It stands to reason the opposite scenario of lower P and higher T (lower density) confers poor solvent power. Co-solvents such as esters, ketones, and short-chain alcohols (especially EtOH due to its low price and relatively low toxicity) can be added. These polar organic molecules modify the solvent power as the since the supercritical fluid can only dissolve non-polar molecules (Krober et Teipel, 2005). This option is still undesirable since it increases the toxicity, composition and risk of interaction with the bioactive compound (Table 1.1).

It has been shown by studies such as those by Thote and Gupta (2005) that the technique is successful in forming microspheres with hydrophilic drugs (dexamethasone in this case). The microspheres give sustained release of the encapsulated compound without any initial burst release. This is specifically desirable for toxic drugs that should remain at a stable plasma concentration such as metformin and pioglitazone. Thereby they can slowly be eliminated without any overloading of the liver and other organs involved in the metabolism of the compound (Sihoven *et al.*, 1999)..

There are many fluids that can serve as solvents such as ethane, propane, acetone, pentane, benzene and so forth in a critical state the properties of these fluids vary greatly and some require dangerous pressures/temperatures to reach their critical states. Some fluids are therefore superior to others with respect to their suitability with respect to encapsulation.

Table 1.1 Strategies and limitations of current to current approaches in supercritical fluid technology for microencapsulation (adapted from Moolman *et al,* 2006).

Approach	**Elaboration**	**Limitations**	**References**
Polymer design	Incorporation of "CO_2-philic" functional groups in new polymers	Need FDA approval for novel polymers	Sarbu *et al*., 2000
Surfactants	The addition of CO_2-soluble surfactants	Need FDA approval for surfactants	Hoefling *et al*, 1993; Yazdi *et al*., 1996
Co solvents	The addition of a co solvent such as methanol or ethanol to increase the solvent power of $scCO_2$	Reintroduces requirement for use of a solvent - many actives are sensitive to solvents	Kazarian *et al*., 1998; Mishima *et al*., 2001; Corrigan and Crean, 2002.

Mixtures of SCFs	The use of a second supercritical fluid to enhance polymer process ability	No obvious second supercritical fluid with desired combination of properties (low/no toxicity, low critical temperature & pressure, low cost, etc.)	
Gas anti-solvent (GAS) technique	Use $scCO_2$ as an anti-solvent to extract the solvent from a sprayed polymer solution and thus precipitate the polymer	Reintroduces requirement for use of a solvent - many actives are sensitive to solvents	Subramanian *et al*, 1999
Use low molar mass and low polarity polymers	These polymers are more amenable to $scCO_2$ processing.	These polymers generally have low mechanical integrity and/or barrier properties	Rindelfleisch *et al*., 1996
Use fats / waxes for encapsulation	Fats, waxes and oils are generally soluble in $scCO_2$	Limited flexibility with regards to properties	Dos Santos *et al*., 2002.

1.3.7 Supercritical Carbon Dioxide

Carbon Dioxide has many properties that make it a very attractive candidate for use as a supercritical solvent. They include but are not limited to;

- Abundancy
- Low Price
- Non-toxicity
- Non-Flammable nature
- Non-corrosive nature
- Low critical temperature
- Low critical pressure

The low T_C and P_C ensures that the whole process does not have to be carried out at extreme pressures (72 bar) and high temperatures (31□C). This adds a measure of safety to the process that is not attainable with other gasses in their supercritical state. $scCO_2$ is described as being a good solvent, this is unfortunately not true in the case of polymers, where it reacts better as a solute, plasticizing the polymers upon depressurization (Dos Santos *et al.,* 2002).

The sub-micron range particles of the solute that are created by the rapid expansion of the gas upon depressurization ensure for a smoother, even coating of the core material. If the solvent does not evaporate quickly enough during depressurization agglomeration occurs between particles, especially fine ones which have strong cohesive forces. Supercritical carbon dioxide is what is known as a selective solvent because it only dissolves non-polar molecules due to its own uncharged nature (Sihoven *et al.*, 1999). It does not have a dipole moment but does however have a quadrupole moment which gives it the ability to interact with groups such as carboxyl, ether and esther (Sarrade *et al.*, 2003).

It creates the abovementioned thinner, more even coating creates extremely small particles (in the submicron range) which have very low cohesive and adhesive forces (Krober and Teipel, 2005). The effectivity of the process using the same amount of product is as a result increased. The low critical temperature and pressure also mean that it is ideal for encapsulation of heat and

pressure sensitive materials (Krober and Teipel, 2005). A large range of materials can therefore be encapsulated easily without risk of toxicity at a very low price. Many materials have already been tested, mostly from the pharmaceutical grade polymers.

1.4. EXCIPIENTS

An excipient is a substance which is pharmacologically inactive and acts as a carrier for bioactive compounds to be delivered to the host. They serve to protect the active ingredient or help with the release of a compound that should be released slowly. Their addition often stabilizes the active ingredient thereby ensuring that the shelf life of the product is increased.

1.4.1 Cellulose and cellulose derivatives

1.4.1.1 Hypromellose

Is used for controlled release of oral medication. Its full name is Hydroxypropyl methylcellulose (HPMC). It is a polymeric substance used as a substitute for gluten in making grain breads. Its chemistry allows it to trap CO_2 bubbles formed by the yeast causing the bread to rise. Its non-toxic nature allows addition to foodstuffs by the Codex Alimentarius (E464). When an aqueous solution of HPMC is heated to a critical temperature it becomes semi-flexible, this temperature is inversely correlated to concentration of exipient and methoxy group. As an exipient it is used a coating as well as a binding agent to give bulk to the tablets (Sarfaraz et Niazi, 2004).

1.4.1.2 Sodium carboxymethyl cellulose

It is an ionic molecule which is produced by reacting cellulose with chloroactetic acid followed by an alkali. It has a high melting temperature of about 135°C and is a linear polyelectrolyte. It rapidly dissolves in water producing clear solutions. At low concentrations in an aqueous solution the molecules are usually linear but become coiled with an increase in concentration and pH. At concentrations exceeding the overlap concentration a thermo-reversible gel is formed (Stelzer and Klug, 1980).

1.4.2 Plant exudates and extracts

1.4.2.1 Gum Arabic

The composition of this substance can vary with climate, season, source, time of exudation along with a list of other considerations but is characterized as a complex mixture of arabinogalactan oligosaccharides, polysaccharides and glycoproteins. Depending on the source the glycan contains different proportions of D-galactose:L-arabinose (in the case of *Acacia seyal*) or vice versa (in the case of *Acacia senegal*). It is branched consisting of β(1-3)-linked D-galactopyranosyl units with side chains of α-L-rhamnopyranosyl, β-D-glucopyranosyl and 4-O-methyl β-D-glucopyranosyl linked at 1,6 linkages. The molecules may be neutral or slightly acidic (Verbeken *et al*, 2003).

1.4.2.2 Gum karaya

It is described as a partially acetylated, complex polysaccharide obtained as a Ca and Mg salt. It is not very soluble with only 10% of the substance dissolving in cold water. This increases to 30% in hot water and 90% if the molecules are deacetylated. It is obtained from the dried and exudates of the *Cochlosperumum* family, most notably the *Sterculia urens* Roxbourg strain in the subcontinent of India (Verbeken *et al*, 2003).

1.4.2.3 Locust bean gum

It falls under a large group of food grade excipients known as the galactomannans. The name comes from the linearly linked (1-4)-linked β-D-mannopyranosyl units with single α-D-galactopyranosyl units connected by 1,6 linkages as side chains. There are different species characterized based on their ratio of D-mannosyl to D-galactosyl. They have different properties based on this. Locust bean gum has a limited water solubility temperature at ambient temperature. It does however swell up until 60°C where it becomes soluble (Seaman, 1980). If heated for 10mins at 80°C it becomes fully hydrated. It is isolated from the endosperm of the seeds of the carob tree (*Ceratonia siliqua*).

1.4.2.4 Mesquite gum

This is the neutral salt of an acidic branched polysaccharide. Some proteins are also binded to the carbohydrate mixture although this amount varies (1-6%). It bears chemical similarity to Gum karaya in that it has comparable solubility. The mixture is however darker and has better film producing qualities. There is a steeper viscosity-to-concentration curve than for gum Arabic. This gum is obtained from the misquite shrub (*Prosopsis* spp.) (Verbeken *et al*, 2003).

1.4.2.5 Soluble soybean polysachcharide

The structure of this substance is not yet fully understood yet it is known that mainly galactose, arabinose and galacturonic acid are present (Picot and Lacroix, 2004). It is an anionic polyelectrolyte like sodium carboxymethyl cellulose because of negative charges the backbone. It has a lower viscosity than locust bean gum, but is soluble in cold water. It has excellent film forming properties but does not gel. It can prevent oxidative stress to oils and to stabilize protein particles at a low pH. It may be used as an emulsifier and as a stabilizer for emulsions. It is extracted from okara. This is the residue after the oil and soy protein extraction from soybeans (Maeda, 2000).

1.4.3 Marine Extracts

1.4.3.1 Alginate

Alginates are marine extracts from seaweed which are considered linear anionic polysaccharides. They are complex charged polymers consisting of (1-4)-linked α-L-glucuronic acid and β-D-mannuronic acid. The solubility is affected by the pH and the presence of competing ions. At a pH above 4 the alginate converts to its corresponding salt, sodium alginate, which is soluble in water. An extended random coil formation results from intermolecular repulsion by the negative charges of each monomeric unit forming very viscous solutions (Draget, 2000). Alginate is subject to rapid degradation and many organisms degrade it but storage in a freezer can increase the shelf life to a couple of years without significant damage. Alginate is harvested are produced commercially from marine brown algae although it is abundant in nature (Champman, 1980).

1.4.3.2 Carrageenan

Carrageenans are polysaccharides extracted from red seaweed species. They are large high molecular weight flexible molecules used in the food industry as a thickening and stabilizing agent (Knutsen *et al*, 1994). Kappa-carrageenan, one of the carrageenan family, is a sulfated polymeric saccharide consisting of alternating linked monomers of 1-3-β-D-galactose-sulphate and (1-4)-3,6,anhydro-α-D-galactose. It is soluble in water above 70□C. In low concentrations it forms a highly viscous solution. The linear chains are sensitive to interactions from ani/kations as they carry a negative charge. This property allows solutions of k-carrageenan to yield gels upon cooling if the right ions are present. K-carrageenan forms very strong gels in the presence of K^+. This occurs over a two step solid to gel transition mechanism where an aggregation of helices take place (Paoletti *et al* 1984).

1.4.4 Microbial and animal Polysaccharides

1.4.4.1 Chitosan

Chitosan is a linear polysaccharide. This sets it apart from the other branched polymers im that it consists mainly of randomly distributed β(1-4) linked D-glucosamine and N-acteyl-d-glucosamine. It is described by Dombard and Dombard (2002) as a non-permanently charged cationic polyelectrolyte. The degree of solubility of chitosan in solutions is related to the degree to which the monomers are acetylated. Only if less than 40% of the residues are acetylated is chitosan soluble in an acidic aqueous medium. Chitosan is derived from chitin which is widely abundant in nature. It has been commercially isolated from fungi but the main source is still the deacytelation of crustacean chitins.

1.4.4.2 Xanthan

This is a high molar mass anionic polyelectrolyte which binds with, Na, K and Ca at a higher pH resulting in its salt form. It consists of β (1-4)-linked-D-glucopyranosyl units linked to one d-glucuronosyl unit between two d-mannosyl units at the C-3 position. Xanthan is soluble in cold water due to its strong anionic nature. Viscosity increases with shear stress and is stable from pH

2-12, but abruptly returns to the original value when the stress is removed. A conversion between a double helical structure and a linear one may occur between 40°C and 80°C over a few hours. Xanthan is harvested from the bacterium *Xanthomonas campestris* in glucose medium by aerobic fermentation (Verbeken *et al*, 2003).

1.4.5 Lipid Based Excipients

1.4.5.1 Compritol

Jagdale *et al* (2011) states that in recent years glyceryl behenates (essentially glycerol ester bonded to behenate groups) have increasingly been used in controlled-release applications by direct compression and other, more recent techniques such as hot-melt coating (Barthelemy *et al*, 1999; Hamdani *et al*, 2003).

Compritol consists of a mixture of mono-, di- and tri-behenates of glycerol in a 18:52:28 w/w ratio. It has a phase transition temperature of between 69□C and 74□C. Its Hydrophillic-Lipophillic Balance (HLB) is 2 (Hamdani *et al.,* 2003). It has also been shown by Jagdale *et al*, (2011) that Compritol does not interact with a highly hydrophilic drug in the solid state, does not form complexes and provides stable sustained release profiles.

1.4.5.2 Gelucires

Gelucires are mixtures of glyceryl mono-, di- and tristearates, some in the form of polethene glycol (PEG) esters of fatty acids. Gelucires are semisolid, inert, waxy ampiphilic excipients used mainly in the delivery of controlled release substances and matrices. They do not change the properties of the biochemical compound they carry and pose no risk of an anaphylactic response from the test subjects and improve the physiochemical properties of the drug (Moes, 1993). Gelucires are characterized by relatively low melting temperatures ranging from 33°C to about 65°C and a wide range of hydrophilic to lipophillic balance (HLB) values of about 1-18 (Sheu *et al*., 2001).

The rule is that the lower the HLB, the slower the matrix releases the bioactive compound. Thus a high HLB allows for quick dissolution of the drug (Ainoui et Vergnaud, 1998; Sheu, 2001).

The advantages of Gelucires compared to other drug delivery systems according an in vitro study by Porter et Charman (2001) are;

- Low melting points
- Prevention of gastric irritation
- Biocompatibility
- Biodegradibility
- No need for solvents
- Absence of toxic impurities

Gelucire® 43/01 is highly lipophillic with an HLB balance of 1 and a melting point of 43°C. The low HLB balance means that it will severely slow drug dissolution time allowing for sustained release of the bioactive compound (Chauhan *et al*., 2005).

1.4.5.3 Vegetal

Vegetal is a collective term for extracts from vegetables comprising of many different types of indigestible starches and fibers. These fibers are not degraded by amylases and so provide a good protective layer for any material it encapsulates. The commercial form of Vegetal is glyceryl di-stearate, a non-polar fatty acid ester of glycerol and two long chain fatty acids. It behaves much as Compritol except in that in contains a mixture of prebiotics that might increase the survivability of the encapsulated cultures.

The complex carbohydrate mixes it contains provide a range a range of benefits among others (Gropper *et al*., 2008; World of Gastroenterology, 14 [p42])

- Regulates blood sugar by shielding carbohydrates from enzymes
- Speeds passage of foods through the digestive system
- Balances intestinal pH
- May reduce risk of colorectal cancer
- Reduces risk of cardiovascular disease

1.5. TECHNIQUES FOR DETERMINATION OF VIABLE NUMBERS OF BIFIDOBACTERIA

The need to distinguish between organisms that are able to remain dividing and metabolising after exposure to different compounds or environments are referred to as viability studies and the need to distinguish these from organisms that are dead is of the utmost importance, especially with respect to studies of the GIT microflora (Bunthof *et al*., 2001).

This is so because the organisms, probiotic or otherwise, pass through different unfavourable environments as they make their journey to the large intestine. If the difference between dead and living cells cannot be distinguished, conditions for ingestion of these organisms or knowing which foods have a positive or negative effect on the growth and survival of these organisms can not be determined.

Probiotic cultures are generally added present in inordinately large numbers, usually 10^7-10^9 cfu's/ml, to negate this effect of organisms dying off and therefore ensuring a number high enough to establish a stable population reaches the lower GIT and therefore be beneficial (Auty *et al*., 2001).

Factors affecting viability of bifidobacteria are as follows (Roy, 2001);

- Oxygen concentration
- pH and fermentation conditions
- Temperature of growth environment
- Strength of selective factor
- Difference in strains
- Presence of other microbes

Viability may be improved by techniques such as microencapsulation and selection of bile-resistant strains.

1.5.1 Selective media

When adequate physical and physiological conditions preside, they will favour the growth of certain bacteria such as bifidobacteria, over that of other bacteria. The selective factor should be incorporated in both the conditions and the medium to provide growth conditions more suited to the bacterium of interest than its competitors. A colouring agent may also be added to provide a more accurate count. Anaerobic conditions are a definite must with the use of all different types of media which stands to reason as they reside among other places in the human GIT.Media traditionally used to culture bifidobacteria are Tryptose-Soy Agar (TSA), Trypticase-Phytone Yeast extract (TPY) which also allow growth of other LAB (Shah, 2000).

The medium most widely use is De Man, Rogosa and Sharpe (MRS) supplemented with 0.05% v/v cysteine-HCl. This agar is most widely used for quality control purposes (Shah, 2000). Different selective antibiotics can be added as an selective factor such as nalidixic acid Neomycin sulphate Lithium chloride Paromycin sulphate (NNLP) agar. For enumeration of bifidobacteria from other LAB growing on the same plate Tryptose Proteose Peptone Yeast extract (TPPY) in addition to Prussian Blue (PB) agar is recommended while Reinforced Clostridial Prussian Blue agar (RCPB) can be used on its own for differentiation (Roy, 2001).

1.5.1.1 Plate count methods

Cell viability in most commercial probiotic products is traditionally assessed by the plate count method where cells actively grow and form visible colonies on solid media (Keer and Birch, 2003). The numbers of viable microorganisms are usually underestimated as fastidious bacteria and viable cells that have lost the ability to form colonies will not be detected (Keer and Birch, 2003). The microbes may also be unevenly distributed in the product or the bacteria could be in clumps or chains which would also result in the same effect (Auty *et al*., 2001).

The method is time consuming, taking about 72h for visible colonies to appear, the presence of oxygen also rendering the growth mute. Strict anaerobic conditions at body temperature are therefore required.

1.5.2 Molecular methods

There are many molecular methods that exist for the enumeration of viable numbers of bifidobacteria. The most popular of these are nucleic-acid amplification tests. These are based on the fact that in an unviable cell the DNA is in a more degraded state due to endogenous endonucleases no longer being down-regulated and therefore having free-rains to digest their respective target sequences. These methods include Polymerase Chain Reaction (PCR) by Kary Mullis in 1983.

1.5.2.1 PCR and RT-PCR

Polymerase Chain Reaction (PCR) and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) targeted at the 16S DNA and 16S rRNA sequences respectively. The techniques amplify these sequences and the resulting nucleic acid bands can be viewed on either agarose gels or as a change in fluorescence. The sequences can also be digested by specific restriction enzymes with known cut sites to give a strain-specific Restriction Fragment Length Polymorphisms (PCR-RFLP's) that are detected by means of an agarose gel (Higuchi *et al*., 1993).

These methods are relatively accurate but are not impervious to error as environmental conditions and background noise caused by nonspecific binding can have a profound effect leading to amplification of spurious products.

1.5.2.2 DGGE and TGGE

Denaturing gradient gel-electrophoresis (DGGE) and Temperature gradient gel-electrophoresis (TGGE) can be used to detect differences in sequence of nucleic acid fragments of the same length. The method uses either pH or temperature to degrade the fragments into single strands, and then measures the ease at which the molecules hybridize. The quicker the molecules hybridize, the higher the degree of similarity (Keer and Birch, 2003).

1.5.2.3 Serological Methods

Microplate assays using different reporters such as enzyme's such as alkaline phosphatase, dye's such as psolarin's or cDFA could be used to detect and discriminate between species. Other methods such as Fluorescent in situ Hybridization (FISH) where a small species specific fragment or 'probe' attached to a fluorochrome is hybridized with a conserved area the DNA of the desired species, ELISA where antibodies bound to reporters bind to membrane or anti-membrane protein or antibodies and flourocytometric methods also be used under hybridization assays (Keer and Birch, 2003).

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CHAPTER 2

ASSESSMENT OF VEGETAL BM 297 ATO AND COMPRITOL E 472 ATO AS CANDIDATE LIPID-BASED FOOD GRADE EXCIPIENTS FOR ENCAPSULATION OF *B. LACTIS* BB12 USING A SUPERCRITICAL CARBON DIOXIDE-BASED PROCESS

2.1 ABSTRACT

A novel method for the encapsulation of probiotics has been developed using a supercritical carbon dioxide ($scCO_2$) based particles from gas saturated solutions (PGSS) process. The excipients used in microencapsulation using $scCO_2$ are usually food grade biolpolymers which negate the need for FDA approval. This study aimed to evaluate the suitability of two new food grade materials with GRAS (generally recognized as safe) status as candidate excipients for encapsulation of probiotic cultures. Compritol E 472 ATO and Vegetal BM 297 ATO were used to encapsulate *Bifidobacterium lactis* Bb12 using $scCO_2$ as a coating material in the PGSS process. The manufactured microparticles were characterized based on morphological properties, particle size distribution as well as encapsulation efficiency. Compritol E 472 ATO was found to be unsuitable due to poor survival of probiotic cultures during the encapsulation process. Vegetal BM 297 ATO was found to be suitable, using mild encapsulation conditions which resulted in better survival of encapsulated probiotics. It's microparticles were found to have an encapsulation efficiency of 97% and a mean particle size of 31 µm. The particles were irregular in shape with a smooth, non-porous surface. Vegetal BM 297 ATO has potential for use in the food and pharmaceutical sectors for controlled release and protective barrier function applications as it has a high bacterial load and produces microparticles small enough to not affect the texture of the food products.

Keywords: Microencapsulation; Supercritical carbon dioxide; Probiotics: *Bifidobacterium lactis*; Vegetal; Compritol.

2.2 INTRODUCTION

The consumption of probiotic organisms is associated with many health benefits (Reid *et al*., 2007). High numbers of probiotic organisms are needed to confer beneficial effects on the host (Salminen *et al*., 1998). The organisms need to be taken in at a minimum of 10^9-10^{10} cfu per day to exert probiotic effects on the host (Fasoli *et al*., 2003). In order to exert beneficial effects these organisms also need to colonize the gut wall epithelium by attaching to the mucus membrane of the gut wall (Salminen *et al*., 1998). If adhesion of the organisms to the gut mucosa is not achieved, the organisms would simply pass through the gastrointestinal tract (GIT). The higher the numbers of probiotic organisms consumed, the higher chance of successful colonization. Probiotic cultures are added to many everyday food products and also have a strong market in the pharmaceutical industry. These cultures undergo many stresses such as light, moisture and oxidation which reduce their viability. Oxygen stress presents a major threat as they are strict anaerobes (Shah, 2007). Loss of numbers of live organisms in a food product occur due to environmental stress factors such as gastrointestinal transit, damage during processing and high storage temperatures (Champagne *et al*., 2005). Studies have shown that numbers of live probiotic organisms in certain functional foods decreased far below recommended levels by the time the product had reached the end of its shelf life (Huff, 2004; Vinderola *et al*., 2004). Protecting the probiotic microorganisms would increase the survival rate of the organisms by protecting them against unfavorable environments.

Microencapsulation of the cells could provide a barrier function against the harsh conditions that probiotic organisms endure. This would result in higher numbers of viable organisms being present in the functional food product at the time of consumption. The microparticles should ideally be below 1mm as the smaller the microcapsule the more palatable it is to the consumer. Problems regarding decreased barrier function have however been reported in microparticles smaller than100µm (Anal and Singh, 2007). It is however reported by Ding and Shah (2009) that smaller size microparticles (<100µm) are desirable based on the fact that smaller capsules have a smaller chance of affecting food texture.

There are different methods of microencapsulation which produce different size ranges of microparticles. Supercritical CO_2 based microencapsulation, such as particles from gas saturated

solutions (PGSS) is currently being explored in probiotics research. The PGSS process uses only $scCO_2$ as a process medium, which is a non-toxic and inexpensive method of immobilizing cells (Moolman *et al.,* 2006).

Supercritical carbon dioxide is what is known as a selective solvent because it only dissolves non-polar molecules due to its own uncharged nature (Yazdi *et al*., 1996; Sihoven *et al*., 1999). Interactions occur only with polymers that have complimentary functional groups such as carboxyl or carbonyl groups (Rindfleisch *et al*., 1996). These react through either Van der Waals, electrostatic, hydrogen bonding and hydrophobic interactions (Hoefling *et al*., 1993; Tilly *et al*., 1994; Sarbu *et al*., 2000). Supercritical CO_2 has the ability to interact with carboxyl, carbonyl, ether and ester groups via Lewis acid-base interactions where CO_2 acts as the lewis acid (Kazarian *et al.,* 1998; Sarrade *et al.*, 2003).

The currently used lipid based excipients offered little or no protection to the encapsulated cultures in addition to providing problems with regard to dispersion of probiotic cells in the matrix and premature melting of the microparticles (Modler and Villa-garcia, 1993; Picot and Lacroix, 2004, Lahtinen *et al*., 2007). Vegetal BM 297 ATO (Glyceryl distearate) and Compritol E ATO (Glyceryl behenate) have potential as food grade polymers for the encapsulation of probiotic cultures because they are stable, hydrophobic, have carbonyl groups, have GRAS status and are relatively inexpensive (Jagdale *et al*., 2011). Both are being used in the pharmaceutical industry as emollients, emulsifiers and thickening agents (Pharmaceutical excipients, 2012) but have not been tested for $scCO_2$ applications, specifically the encapsulation of probiotics. The aim of this current study was to evaluate the suitability of Vegetal BM 297 ATO and Compritol E 472 ATO as excipients for use in probiotic cell immobilization using $scCO_2$.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial Culture

Bifidobacterium lactis Bb12 (CHR- Hansen) was obtained in freeze-dried form. The culture was stored at -4°C.

2.3.2 Excipients

Biogapress Vegetal BM 297 ATO and Compritol E 472 ATO (Gattefosse, France) were obtained in powder form and stored in sealed containers at 4°C.

2.3.3 Differential Scanning Calorimetry (DSC)

The respective melting temperatures of Compritol E 472 ATO and Vegetal BM 297 ATO were determined by differential scanning calorimetry (DSC) at the Council for Scientific and Industrial Research (CSIR). A DSC Q2000 (TA Instruments), calibrated with indium and zinc, was used to perform the DSC analysis. A heating rate of 10°C/min was used in a nitrogen atmosphere, with a flow rate of 25 ml/min. The temperature range was 0 to 120°C. Aluminum sample pans were used. The sample masses, which were accurately determined on an analytical balance, ranged between 2 and 5 mg.

2.3.4 Encapsulation of Bacteria

Encapsulation was accomplished using the PGSS technique (Separex Equipments, France). All equipment was wiped with 70% alcohol and allowed to dry before contact with materials. 8 g of Vegetal BM 297 ATO and Compritol E 472 ATO were weighed off respectively and added to the freeze dried *B. lactis* Bb12 culture (2 g). The freeze dried culture was analyzed and found to contain 1.5 x 10^9 cfu/g. The blend was then mixed by stirring, and was immediately transferred to a pre-heated 1 liter mixing chamber (50˚C). The chamber was sealed, flushed and pressurized with sterile filtered CO_2 (99.995 % purity, Air Products) up to a pressure of 300 bar, with the temperature being adjusted to 50°C for Vegetal BM 297 ATO and 70°C for Compritol E 472 ATO. The material was left to equilibrate for 3 h with intermittent stirring (200 rpm), being

activated after 1 h. The CO_2 was then removed via depressurization and the encapsulated product was collected directly from the mixing chamber.

2.3.5 Determination of Viable Numbers After Encapsulation

Vegetal and Compritol microparticles (100 µg) were suspended in 900 µl of absolute ethanol (Illovo) and vortexed for 30 s to release the encapsulated cells. The mixture was spun down in a microcentrifuge and the supernatant removed. The pellet was then re-suspended in ¼ strength Ringer's solution up to a final volume of 1 ml. A serial dilution was then performed and plated out on MRS (De man, Rogosa and Sharpe) agar (Merck) supplemented with 0.05% v/w cysteine hydrochloride using the pour plate method. The plates were incubated in anaerobic jars at 37°C for 72 h with Anaerocult A gaspaks (Merck). Anaerocult C test strips (Merck) were used to confirm the absence of oxygen.

2.3.6 Determination of Encapsulation Efficiency

The encapsulation efficiency (EE %) was measured by determining the amount of bacteria entrapped into the microparticles. Nine hundred microliters of absolute ethanol (Illovo) was added to 100 µg of entrapped bacterial cells in a 1.5 ml Eppendorf tube. The mixture was vortexed for 15 s and centrifuged at 5000 rpm for 30 s. The supernatant was removed and the pellet resuspended in 900µl ¼ strength Ringers solution up to a final volume of 1ml. A serial dilution up to 10^{-9} was performed and 100 µl aliquots were plated out in Petri dishes containing De Man, Rogosa and Sharpe (MRS) medium (Merck) supplemented with 0.05 % cysteine hydrochloride using the pour plate method. Plating was carried out in triplicate. The plates were incubated at 37 ° C for 72 h in anaerobic jars with Anaerocult C test strips (Merck) for confirmation of anaerobic condition. Viable numbers were determined by plate count method using a colony counter. The mean of 3 triplicate counts was used.

Encapsulation efficiency was calculated using the equation:

$$EE\% = \frac{\text{(Total bacteria released from microparticles)}}{\text{(Total initial bacteria in encapsulation mix before encapsulation)}} \times 100$$

2.3.7 Particle Distribution

Particle size distribution and mean particle size was determined using a Malvern Mastersizer (Mastersizer 2000, Malvern Instruments, U.K.). Two identical samples of the Vegetal microparticles created at 50°C with a ratio of 3 g *B. lactis* to 7 g Vegetal BM 297 ATO were analysed in triplicate and the means of the three readings were recorded. The particles were dispersed in water before measurement at 25 ˚C.

2.3.8 Scanning Electron Microscopy

The encapsulated bacterial powder (100 µg) was sputtered with gold under argon atmosphere (Emitech K550X, Ashford, UK). The particles were transferred to the aluminum pins using double sided adhesive tape. Viewing was done using a JSM-840 microscope (JEOL, Tokyo, Japan).

2.3.9 Transmission Electron Microscopy

The microparticles (100 µg) suspended in 1/4 strength Ringers solution were spun down at 5000rpm for 2 m (Minispin). The supernatant was removed and pellet was then fixed in 2.5% glutaraldehyde solution in a 0.075 M phosphate buffer (pH 7.4). The pellet was then rinsed three times in phosphate buffer and fixed in 0.5% osmium tetroxide. The sample was rinsed with distilled H_2O and dehydrated alcohol in steps (50, 70, 90, 100%) before being infiltrated with Quetol epoxy resin (30% for 1h, 50% for 1h, 100% for 2h) and allowed to polymerize for 39h at 60°C. Ultrathin sections were cut and stained with aqueous uranyl acetate. A counterstain with lead acetate was performed and the sections were rinsed in dH_2O. Then 0.5 µm monitor sections were cut and stained in Toluidine blue as per the UP protocol for preparation of biological samples for transmission electron microscopy. The preparations were viewed under a JEOL JEM-2100F (JEOL, Tokyo, Japan).

2.4 RESULTS AND DISCUSSION

2.4.1 Determination of Encapsulation Condition Parameters

The encapsulation conditions for the two excipients was determined by taking into account the melting temperature of the compound, the conditions that would harm the probiotic cultures and the conditions needed for CO_2 to reach a supercritical state. CO_2 reaches a supercritical state at a pressure of 72 bar and 31˚C (Dos Santos *et al.,* 2002). For the excipient to fully liquefy in the scCO_2, the temperature must be equal to or higher than its melting temperature in the supercritical CO_2 medium. The higher the temperature inside the reactor chamber the higher the chance of loss of live probiotic cultures. It is thus desirable to have an excipient that has a low melting temperature so that the encapsulation conditions are less harsh on the probiotic organisms. Pressure affects melting temperature (Brown *et al.,* 2000).

In the case of CO_2 an increase in pressure causes increased density of the CO_2 which causes more CO_2 to be absorbed into the compound. The more CO_2 absorbed into the compound, the greater the depression of melting temperature. This effect lasts until a critical pressure is reached after which the compressive force becomes the overriding effect. When this occurs the melting temperature will start to increase again (P.W. Labuschagne, personal communication). Bifidobacteria can withstand immense pressures with 10^4 bar leading to a 2 log cycle reduction in numbers of live organisms (Warmińska-Radyko *et al.,* 2001). The encapsulation pressure was therefore chosen as 300 bar, which is the maximum pressure the PGSS equipment can withstand. The melting temperature at atmospheric pressure (1.014 bar) for Compritol E 472 ATO was determined to be 75˚C (Fig 2.1) and 60˚C for Vegetal BM 297 ATO (Fig 2.2). The temperatures needed to liquefy the excipients at a pressure of 300 bar was determined to be 70˚C and 50˚C for Compritol E 472 ATO and Vegetal BM 297 ATO, respectively.

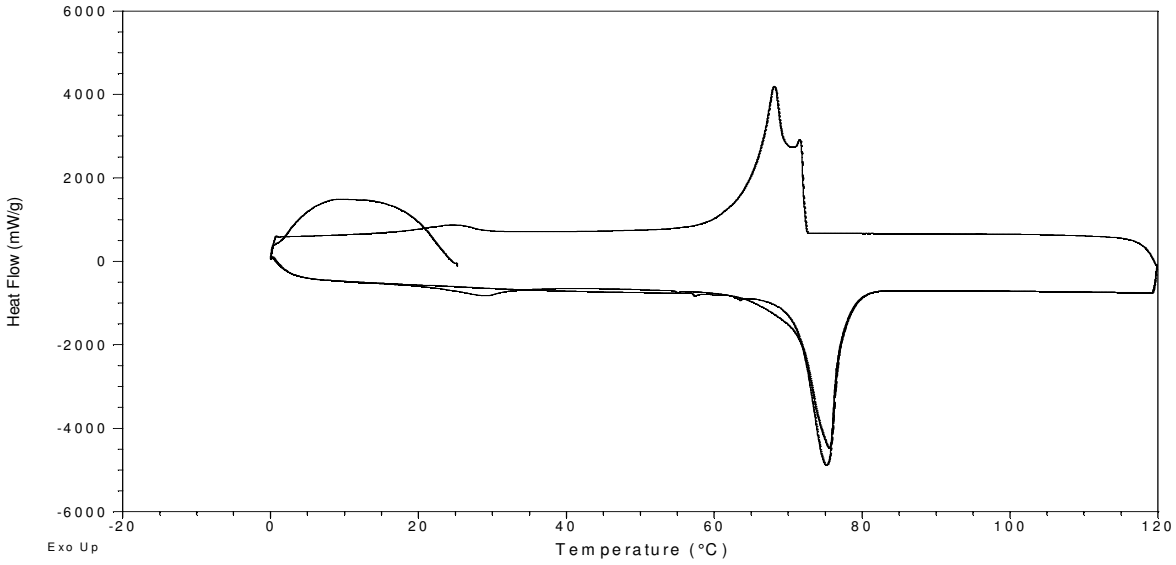


Figure 2.1 Differential scanning calorimetry graph of Compritol E 472 ATO.

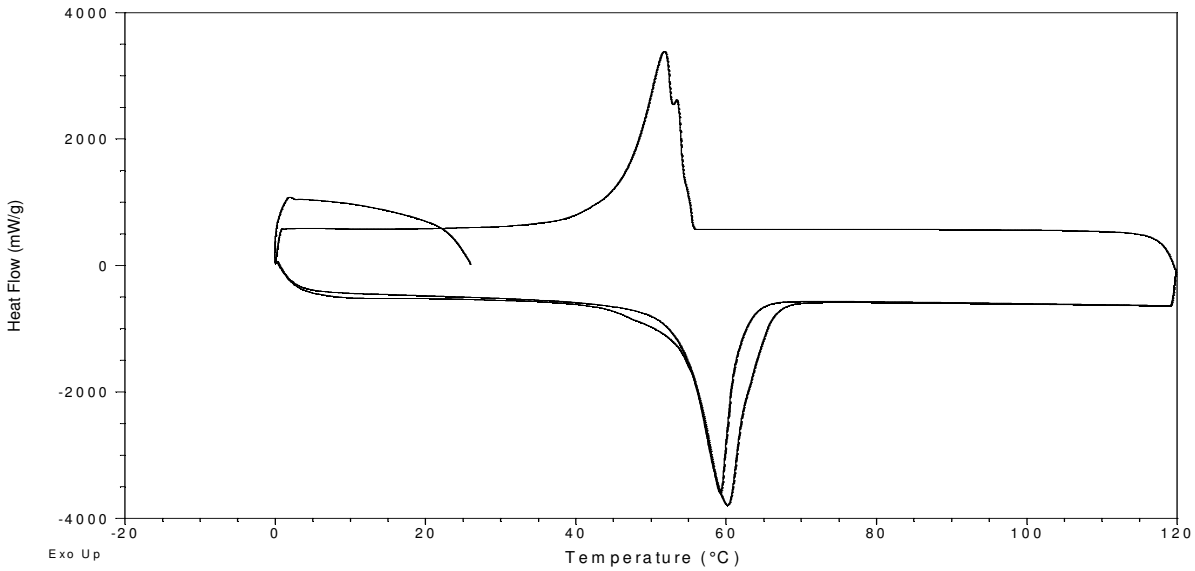
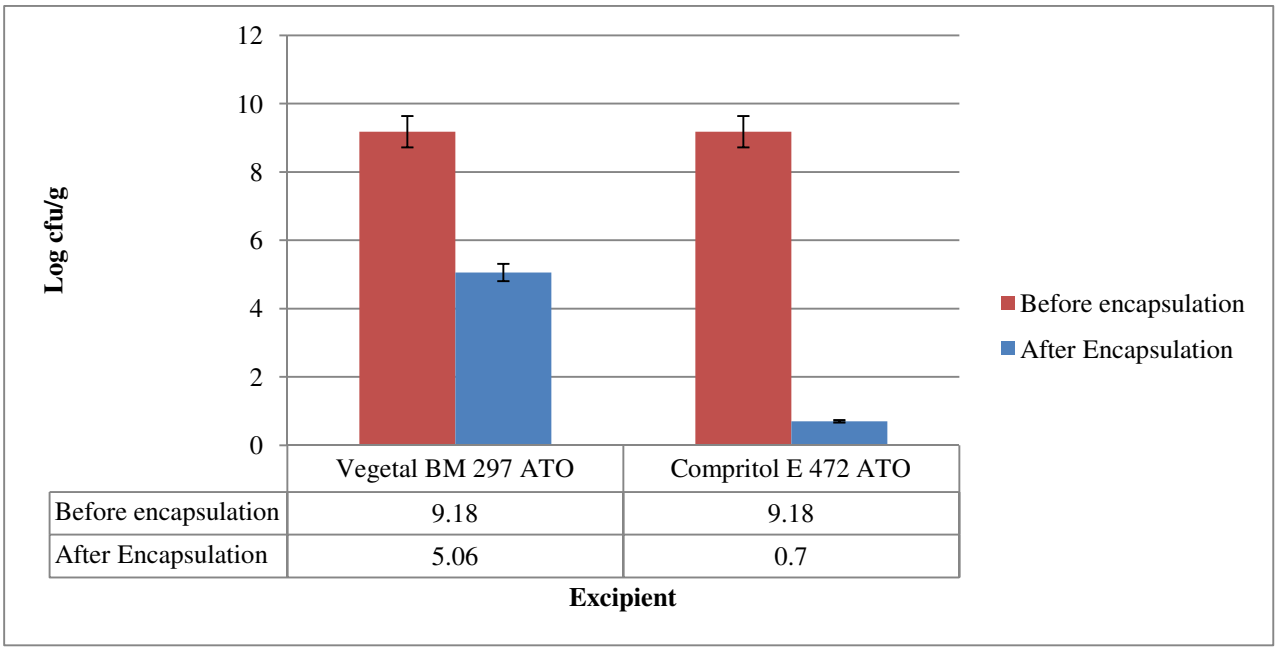


Figure 2.2 Differential scanning calorimetry graph of Vegetal BM 297 ATO.

2.4.2 Preliminary viability testing

The probiotic cultures were encapsulated with Compritol E 472 ATO and Vegetal BM 297 ATO to asses if they could survive the conditions determined in 2.4.1. The higher mixing chamber temperature of 70˚C needed to liquefy the Compritol E 472 ATO resulted in an 8.5 log cycle decrease in numbers of live *B. lactis* Bb12 (Fig 2.3) On the other hand Vegetal BM 297 ATO which liquefied at a lower temperature of 50˚C resulted in only a 4 log cycle reduction in viable numbers of probiotic organisms. This loss in numbers of viable cultures was considered too high. The conditions that would result in fewer losses of live probiotic organisms were determined.



	Vegetal BM 297 ATO	Compritol E 472 ATO
Before encapsulation	9.18	9.18
After Encapsulation	5.06	0.7

Figure 2.3 Levels of viable *B. lactis* Bb12 culture before and after encapsulation with Vegetal BM 297 ATO and Compritol E 472 ATO.

A decrease of less than 1 log cycle of viable numbers of *B. lactis* is desirable as the higher the numbers of cells that survive the encapsulation process, the higher the EE% will be. The reduction in viable numbers caused by the high encapsulation temperature conditions needed to liquefy Compritol E 472 ATO was too high to warrant further testing. Attempts to use mixing chamber temperature conditions resulted in poor plasticization and hence poor encapsulation of bacteria. This indicated that a higher temperature was critical to successful utilization of this

material as an encapsulation coat using supercritical conditions. Therefore Vegetal BM 297 ATO which resulted in less reduction of live organisms was used in further optimization tests.

2.4.3 Optimization of encapsulation conditions using Vegetal BM 297 ATO

It was decided that different formulations of bacteria to excipient could yield better results. A ratio of 3 g *B. lactis* Bb12 to 7g Vegetal was chosen. The ratio was chosen on the basis that the less bacterial cells needed in the final formulation the less expensive the food additive would ultimately be, as probiotics are expensive and unlikely to be covered by medical insurance in the near future (Heidebach *et al*., 2012). It has been suggested that at higher load of cells per microparticle, the bacteria are better protected against harsh environments due to cell-cell interactions (Klayraung *et al*., 2009).

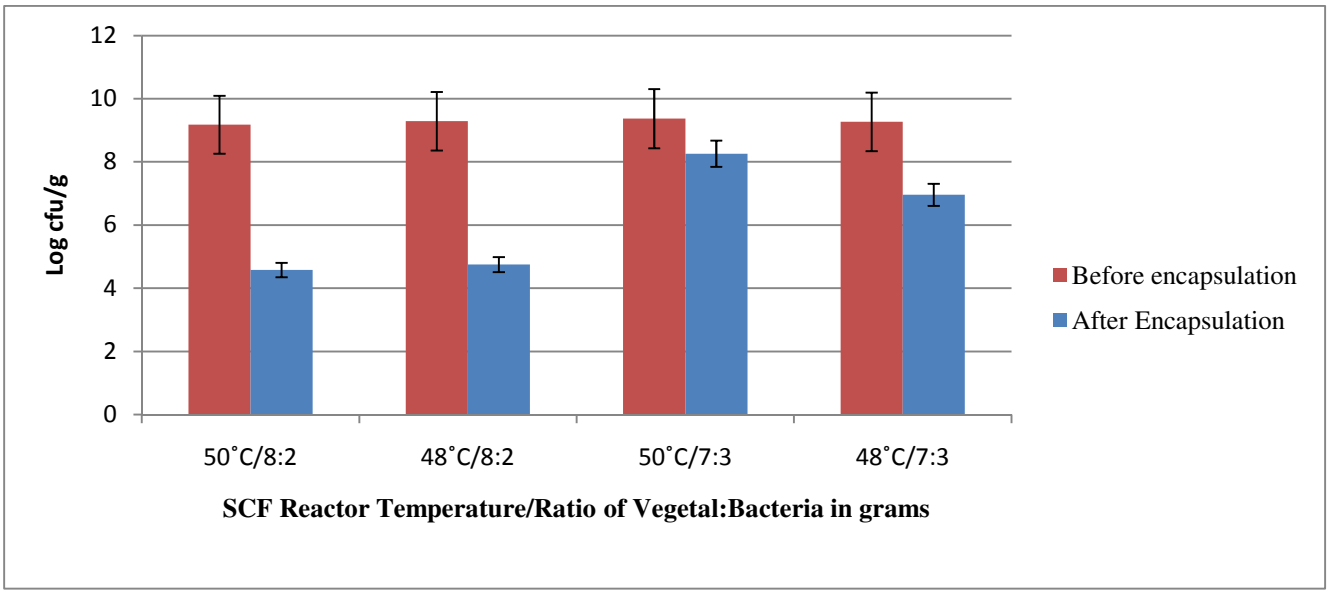


Figure 2.4 Levels of viable *B. lactis* Bb12 culture before and after encapsulation with Vegetal BM 297 ATO and Compritol E 472 ATO under different processing conditions and formulations.

The product obtained directly from the mixing chamber with encapsulation done at 50˚C with 8:2 ratio of Vegetal to bacteria was a solid light yellow mass with a slight buttery odour. When ground in a mortar and pestle, yellow grains with uniform color and consistency was observed. The product formed by encapsulation done at 48˚C was a fine white powder containing solid

yellow clumps. After grinding in a mortar and pestle there was a fine pale yellow powder with larger yellow granules present. Uniform texture is desirable in a food additive as differences could affect the sensory properties of the food (Heidebach *et al*., 2012). The properties of the product obtained at 50˚C process temperature, is therefore more desirable due to its even texture. The viable counts in both formulations before processing were ~9.2 log cfu/g (Fig 2.4).

The decrease in numbers of viable cultures was 4.54 and 4.6 log cycles for reaction products obtained at 48˚C and 50˚C respectively. An EE of 49% and 51% was achieved respectively. This shows similar losses at both temperatures.

The visual characteristics of the two products suggest that poor plasticization occurs at 48˚C as some of the Vegetal powder did not dissolve. This implies that testing of process temperatures lower than 48˚C is unnecessary as a higher temperature is needed for complete plasticization. The need for testing of process temperatures above 50˚C is not necessary as the material completely liquefies at this temperature. Higher temperatures will expose the probiotic organisms to harsher conditions which will lead to loss of viable cells. It was decided that a change in reactor temperature was not advisable as 50˚C is the minimum needed for complete plasticization of the excipient.

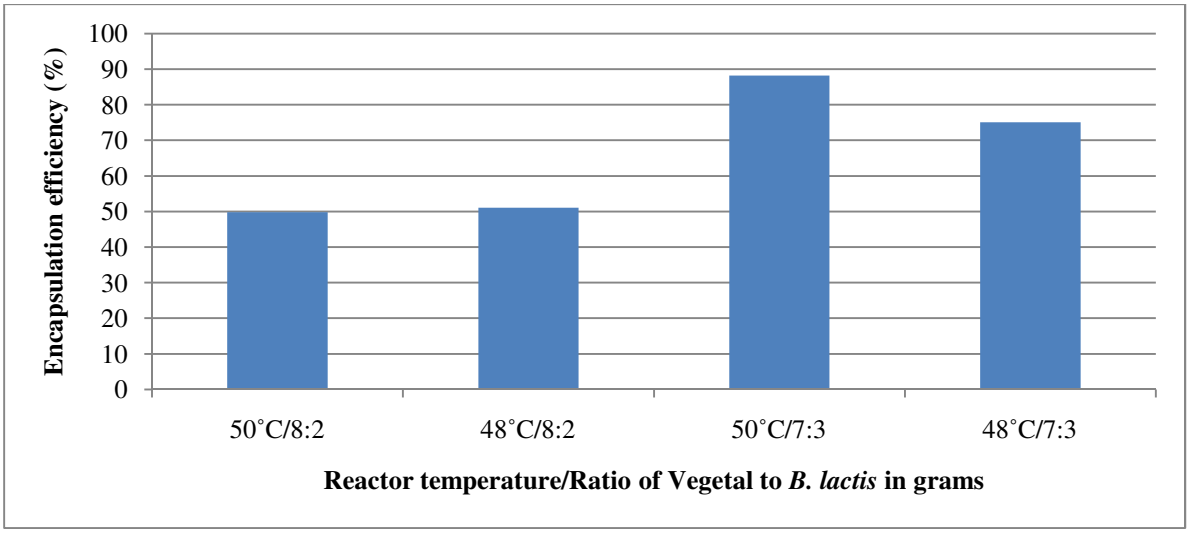


Figure 2.5 Encapsulation efficiencies of different formulations under different encapsulation conditions

A 7:3 ratio of Vegetal BM 297 ATO to *B. lactis* Bb12 proved successful enough that the addition of more bacteria would have been unnecessary. The new encapsulation mixture was plasticized at both 48˚C and 50˚C to compare the properties of the products obtained from the mixing chamber to the previous formulation. The results were similar. The product obtained from the encapsulation at 50˚C was a solid brittle yellow mass that was milled into a fine yellow powder. The product obtained from the mixing chamber at 48˚C contained undissolved Vegetal BM 297 ATO powder with clumps of brittle yellow granules.

The numbers of viable cultures present in the mixture before processing was ~9.3log cfu/g (Fig 2.4). The decrease in numbers of viable cells was 2.3 and 1.1 log cycles for the processing done at 48˚C and 50˚C respectively. An encapsulation efficiency of 75% and 88% was achieved (Fig 2.5). The microparticles obtained from the higher process temperature of 50˚C showed improved survival over the product obtained at 48˚C. The particles obtained from the encapsulations done at 50˚C with a ratio of 3 g of bacteria to 7 g of Vegetal BM 297 ATO performed showed the highest numbers of viable bacteria after processing with an encapsulation efficiency of 88% (Fig 2.5). This method was used to manufacture the microparticles to be characterized.

2.4.4 Particle distribution and encapsulation efficiency

The microparticles of the samples processed at 48˚C and 50˚C showed similar bell-curve distribution patterns with a clear peak at 30-32 µm (Fig 2.6). Both also produced microparticles with an average D_{50} of 31.66 which means 50% of the particles were smaller than 31.66 µm. The average D_{90} of 108.36 also shows that only 90% of the particles were smaller than 108.36µm. Inversely it shows that only 10% of the particles are larger than 108.36µm.

The raw data shows that only 13% of the particles were larger than 100µm. The particles had a mean diameter of 31+-21µm (Mean +-standard deviation, n=6). This is desirable as smaller particles have a smaller chance of affecting the foods' texture and will be more palatable to the consumer (Hansen *et al.*, 2002; Desai and Park, 2005). It has been suggested that particle size should ideally be below 350µm (Roibitaille *et al.*, 1999) but newer research suggests particle sizes below 100µm for stability, easier handling and storage of cultures and limited effects on food texture as larger microparticles can affect mouthfeel (Doyleres and Lacroix, 2005; Ding and Shah, 2009).

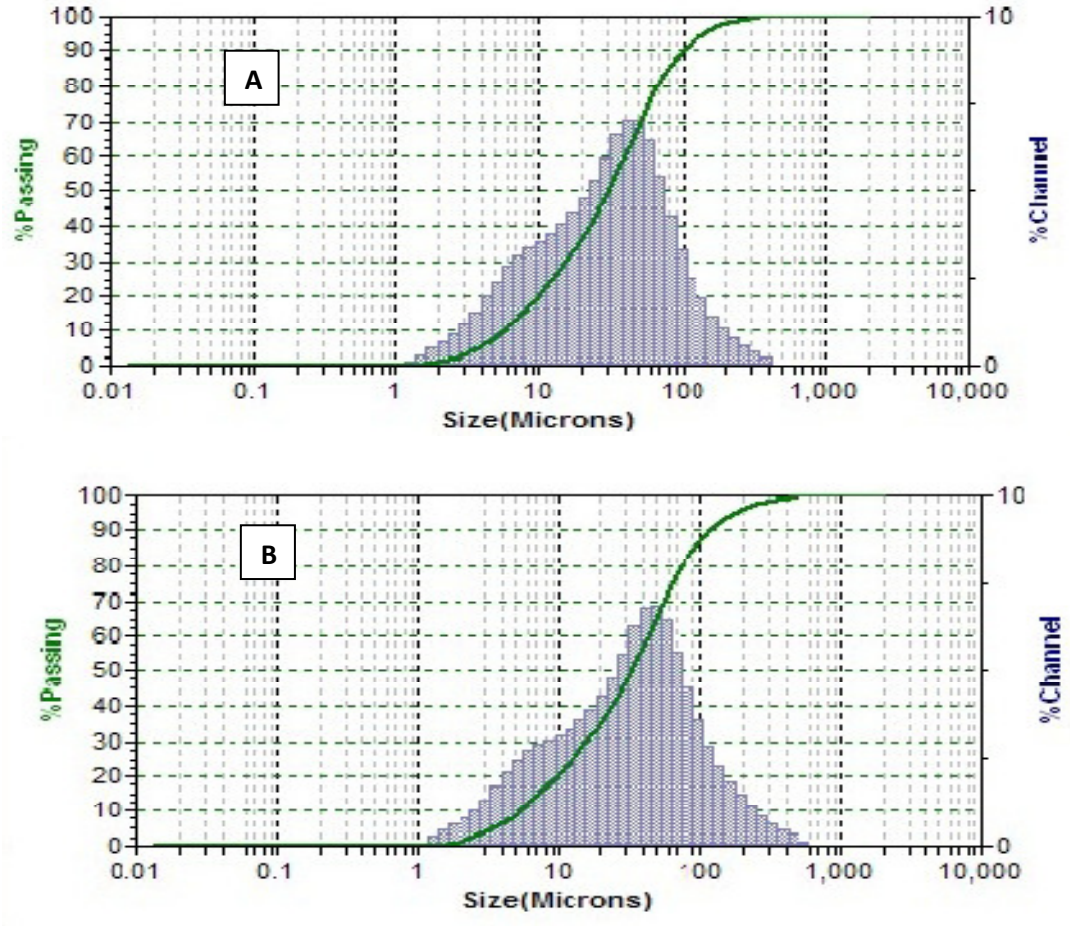


Fig 2.6 Particle size distribution of Vegetal microparticles encapsulating *B. lactis* Bb12 encapsulated at (A) 48°C and (B) 50°C.

Other studies investigating microencapsulation of probiotics using supercritical carbon dioxide reported larger particle sizes. A study by Mamvura (2012) reported microparticles with a mean size of 166 µm when using an interpolymer complex of poly-(vinylpyrrollidone)-poly-(vinylacetate-co-crotonic acid). The particles were reported to be porous, which is thought to be caused by the CO_2 escaping from the microparticles during the depressurization process, or hydrophobic interactions in the matrix (Kazarian *et al*, 1998; Mishima *et al*., 2001; Corrigan *et al*., 2002; Moolman *et al*., 2006). These pores are undesirable as it will expose the entrapped probiotic cells to the harsh environment it is supposed to shield it from (Heidebach *et al*., 2012).

The presence of pores could also interfere with the release of the bacteria (Ravi *et al*., 2008). The Vegetal microparticles are more desirable based on a smaller size and lack of pores. Other methods of encapsulation of probiotics also report microparticles larger than what was found in this experiment. Alginate encapsulation of 3 Bifidobacterial strains have resulted in mean particle sizes of 250 µm (Gobletti *et al.*, 1998), 500-1000 µm (Godward and Kailasapathy, 2003), 2000-3000 µm (Muthukumarasamy and Holley, 2006) and 200-300 µm (Ozer *et al*., 2009). Various strains of bifidobacterial *spp*. were encapsulated using denatured whey protein solutions by Picot and Lacroix (2004). The generated microparticles were hydrophobic and had a comparable size range of 3-75µm.

Percentage encapsulation efficiency was also measured for both samples and shown to be 88.1% (Table 2.2). This value is lower than the 96% reported by Mamvura (2012) using an interpolymer matrix excipient under similar encapsulation procedures. A high EE is desirable as it indicates minimal loss and complete release of the probiotic cells. In a study by Annan *et al.* (2008), *B. adolescentis* was encapsulated in small alginate-coated gelatin microparticles. The microparticles had a comparable size of 50 µm. An EE of 41-43% was obtained. The strong stability of the covalently cross-linked gels were stated as a possible reason for a lowered EE% due to incomplete release of cells from the matrix. Incomplete release of the cells from the matrix will result in a underestimation of the number of viable cells. This will in turn lead to a lower EE% value. Encapsulation with Vegetal produced a lower EE than similar studies using $scCO_2$ but far higher than other encapsulation methods.

Table 2.2 Particle charactacteristics of 2 batches of Vegetal BM 297 ATO microparticles

Mixing Chamber Temperature	D_{10}	D_{50}	D_{90}	Mean Size (µm)	Paricle shape	Encapsulation Eficciency (EE)
50˚C	5.62	30.38	98.62	30.38	Irregular	88.1%
50˚C	5.24	32.94	118.1	32.94	Irregular	88.1%

A study by Moddler and Villa-Garcia (1993) revealed that encapsulation of probiotics in butterfat afforded no protective measure when stored in yoghurt. Dispersion of probiotic cells in

lipid-based microparticles has been described as a difficult technological task due to premature melting of the capsule in elevated temperatures and uneven dispersion of probiotic cells in oil (Moddler and Villa-Garcia, 1993; Picot and Lacroix, 2004). As the melting point of Vegetal BM 297 ATO is 60°C it is a solid powder under normal conditions and will not melt. The fact that it is a solid also means that no separation problems due to different densities or phases will occur. Phase separation was listed as a concern by Lahtinen *et al*. (2007) stating that lipid-based microparticles may be limited to solid foods. The higher melting temperature and solid nature of the Vegetal BM 297 ATO microparticles are therefore desirable as these listed problems will not arise.

2.4.5 Particle characterization by scanning and transmission electron microscopy

SEM revealed that the Vegetal BM 297 ATO microparticles containing *B. lactis* Bb12 had an irregular shape and a smooth outer surface (Fig 2.7 B and C). The smooth coating and particle size are said to be a product of the supercritical carbon dioxide encapsulation process (Krober and Teipel, 2005). Non-spherical microparticles created using $scCO_2$ have been reported (Thantsha, 2007; Mamvura, 2012). The use of other encapsulation techniques emulsion, extrusion and spray drying resulted resulted in spherical microparticles (Adhikari *et al*., 2000; Sultana *et al.*, 2000; Iyer and Kailasapathy, 2005; Zhao *et al.*, 2008). Irregular shape is undesirable as it will affect the dispersion of microparticles in the final food product. Non-spherical particles are reported to be more prone to uneven distribution in a foodstuff (Zhao *et al*., 2008). The particles reported in the literature were consistently larger than the Vegetal BM 297 ATO microparticles containing *B. lactis* and had lower encapsulation efficiency. The high encapsulation efficiency and small size of the Vegetal BM 297 ATO microparticles containing *B. lactis* Bb12 warrants further testing.

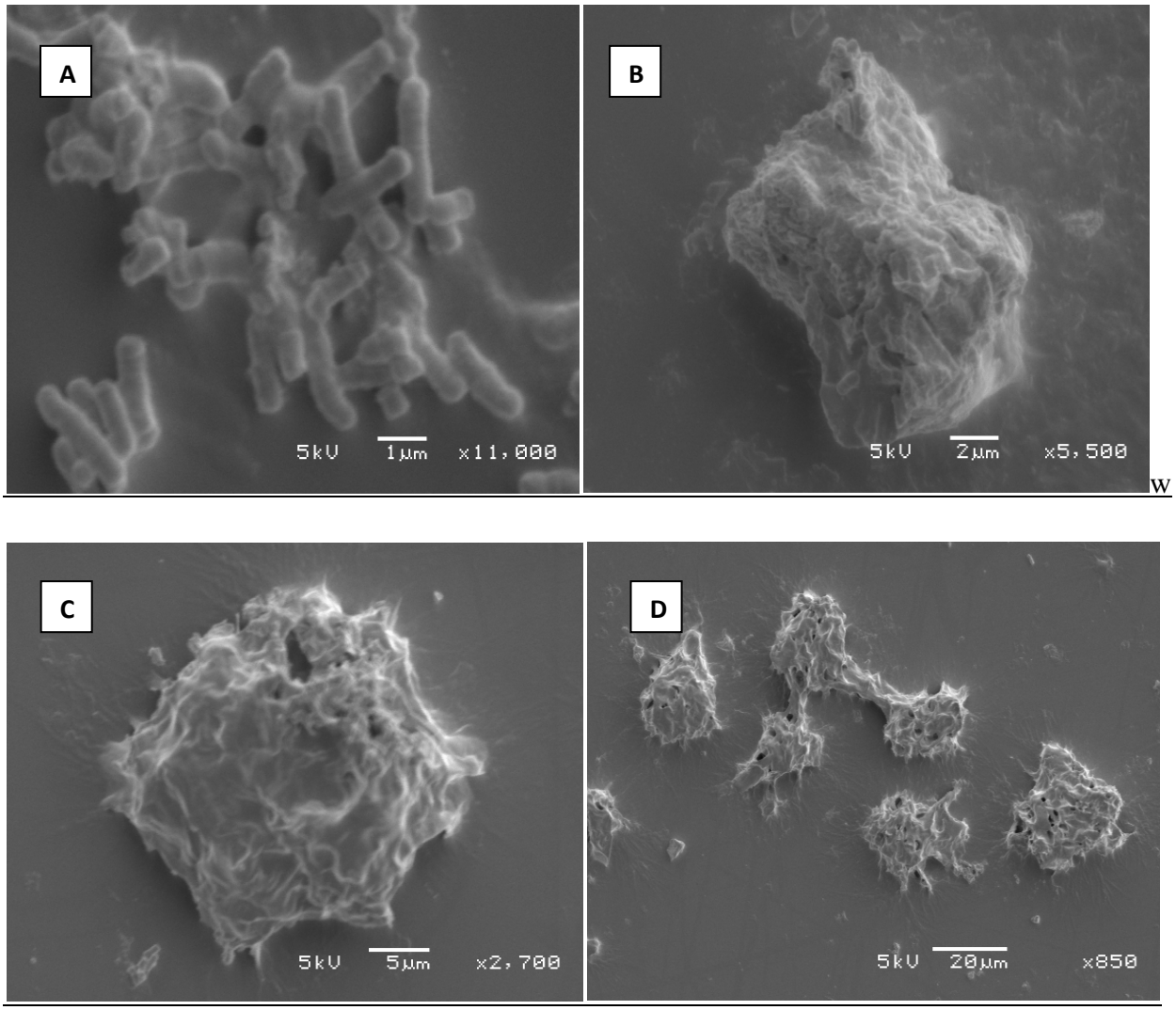


Figure 2.7 SEM images of A : Unencapsulated *Bifidobacterium lactis* Bb12 cells, B and C: a single Vegetal microparticle and D: several Vegetal microparticles

To study the release of *B. lactis* from the Vegetal BM 297 ATO microparticles they were exposed to simulated instestinal fluid (SIF, pH=6.8) and viewed under the SEM. The particles were found to dissolve quicker in a high pH environment which resulted in release of *B. lactis* entrapped within the matrix. A close up of the released material showed that it contained *B. lactis* cells (Fig. 2.8 A). These cells showed characteristic bifidobacterial morphology indicating that there was no observable change.

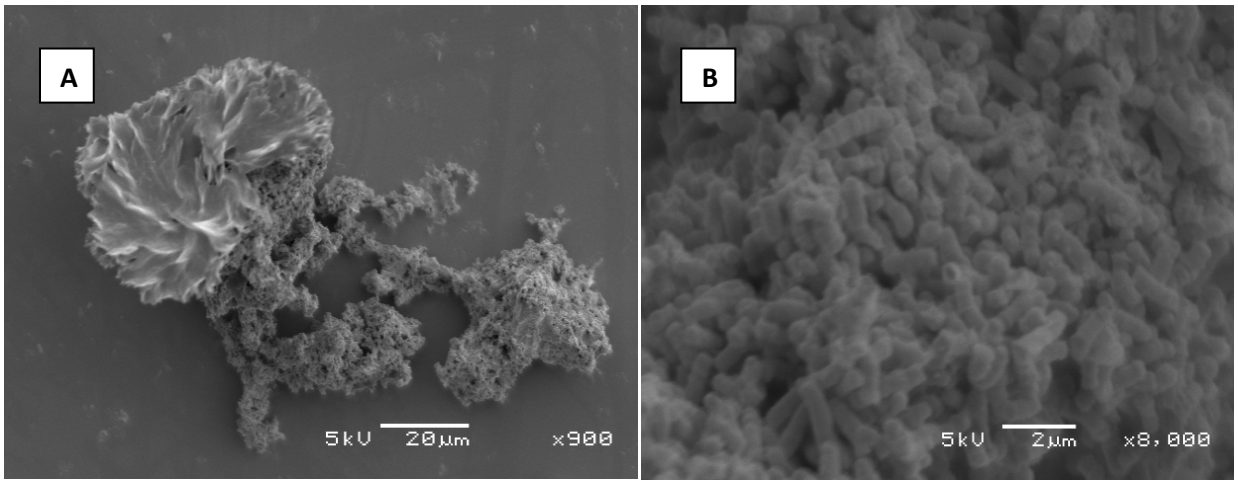


Figure 2.8 SEM micrographs of A: Vegetal microparticles containing *B. lactis* after exposure to SIF for 2 hours and B: A magnified image of the released contents

To confirm this, an ultrathin cross-section of a group of microparticles was taken using a transmission electron microscope (Fig 2.9 A). Visual examination shows that the microparticles are filled with high numbers of bacteria (Fig 2.8 B, Fig 2.9 A and B). Probiotic organisms are 1-5 µm in size (Anal and Singh, 2007). The TEM image shows that the *B. lactis* organisms are tightly packed, which suggests that a high bacterial load was achieved.

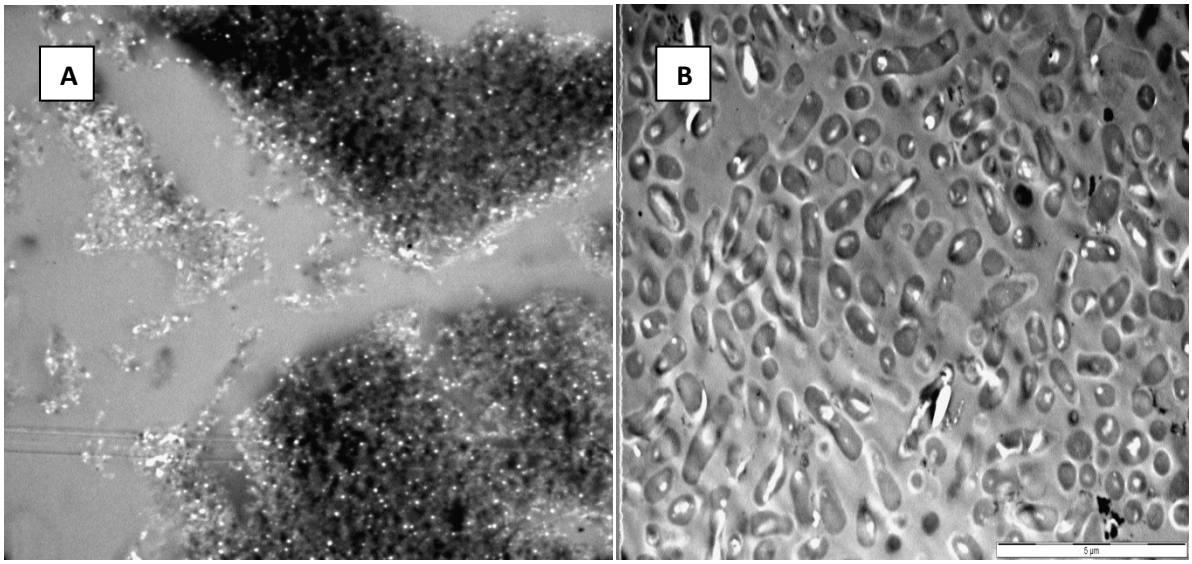


Figure 2.9 TEM micrographs of ultrathin cross-sections of *B. lactis*/Vegetal microparticles.

The average diameter of the Vegetal BM 297 ATO microparticles was 31 µm (Table 2.2). Taking an average particle diameter of 30 µm, the estimated volume is $4/3\pi(15\mu m)^2=942\mu m^3$.

The average volume of a *B. lactis* cell (Cylindrical cell where radius=0.5 µm and length=2 µm, Picot and Lacroix, 2004) is 1.6 μm^3. This suggests that an average Vegetal BM 297 ATO microparticle will contain 177 *B. lactis* cells if the total volume of the particle is 70% Vegetal BM 297 ATO assuming even distribution throughout the matrix. This even distribution throughout the matrix can be observed (Fig 2.9 B).

2.5 CONCLUSIONS

The results of this study show that the Compritol E 472 ATO cannot be used for production of microparticles containing *B. lactis* Bb12, as it requires the use of temperatures that are detrimental to the survival of the probiotic bacteria. However, the results indicate the suitability of Vegetal BM 297 ATO as a suitable lipid-based excipient for use in microencapsulation applications due to its low melting point which does not result in a significant decrease in numbers of viable probiotic cells. Furthermore, the Vegetal BM 297 ATO microparticles have a small size and high EE%, both of which are desirable properties for microparticles used in food applications. These results warrant more research into the performance of Vegetal BM 297 ATO microparticles in protecting the enclosed probiotics during exposure to simulated gastrointestinal environment as well as during storage of products.

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CHAPTER 3

SURVIVAL OF *BIFIDOBACTERIUM LACTIS* Bb12 ENCAPSULATED IN VEGETAL BM 297 ATO MICROPARTICLES DURING STORAGE AT DIFFERENT TEMPERATURES AND IN SIMULATED GASTROINTESTINAL CONDITIONS

3.1 ABSTRACT

This study investigated the effect of microencapsulation in Vegetal BM 297 ATO on the survival of *B. Lactis* Bb12 during simulated gastrointestinal transit and shelf life under different conditions. To assess shelf life stability different samples of both the non-encapsulated and encapsulated cell samples were stored at 4˚C and room temperature (22˚C) for 12 weeks. Stored samples were analyzed weekly to determine viable numbers. To assess survival in simulated GIT fluids, free and encapsulated *B. lactis* Bb12 were subsequently exposed to simulated gastric fluid (pH=2) for 2 h and then to simulated intestinal fluid (pH=6.8) for 6 h at 37˚C. Samples were taken at 0. 0.5, 2, 4, 6 and 8 h and analysed for viable numbers. All samples were diluted, pour-plated using MRS-agar supplemented with 0.05% cysteine hydrochloride. The plates were then incubated anaerobicaly at 37˚C for 72 h. Vegetal encapsulation did not extend the shelf life of *B. lactis*. Counts reached the minimum threshold for numbers of live probiotic organisms at 14 and 48 days at room temperature and 4˚C respectively. The Vegetal BM 297 ATO microparticles continuously released *B. lactis* as the matrix dissolved. The viable numbers of cells released from the Vegetal BM 297 ATO microparticles were higher than those of the non-encapsulated cells at the end of exposure to simulated GIT fluids, indicating a protective effect by the Vegetal BM 297 ATO matrix. These results show that Vegetal BM 297 ATO microparticles did not increase the shelf life of *B. lactis* Bb12, but offered them protection from harsh gastrointestinal environments.

Keywords: Simulated gastric fluid, Simulated intestinal fluid, Vegetal, Probiotics, *Bifidobacterium lactis* Bb12, microencapsulation.

3.2 INTRODUCTION

Probiotic cultures are already common additions to many products used daily in most households and already have a strong market for themselves in the pharmaceutical industry. The range of benefits associated with the use of probiotics include lowered blood pressure and cholesterol, improvement of immune functioning, aided digestion of fiber and thereby stimulation of bowel movement and neutralization of carcinogenic compounds in the gut (Gilliland and Walker, 1990; Brady *et al*., 2000; Hickson *et al*., 2007; Reid *et al*., 2007).

In order to exert these beneficial effects these organisms need to colonize the intestinal epithelium by attaching to the mucous membrane of the gut wall (Salminen *et al*., 1998). If adhesion is not achieved, the organisms would simply pass through the intestines. The probiotic organisms also compete with other established gut microorganisms for nutrients and adherence sites (Lian *et al*., 2003). The higher the numbers of live probiotic organisms present in the colon at time of colonization, the higher the chance of successful establishment of a population.

The organisms need to be taken in at a minimum of 10^9-10^{10} cfu per day to exert probiotic effects on the host (Fasoli *et al*., 2003). The foodstuff containing the probiotic organisms should therefore contain enough live cultures at the time of consumption so that an adequate amount will survive to reach the intestine for colonization. Studies have reported that the number of viable probiotic cultures declined to unacceptable levels by the end of product shelf life. The levels of live cultures were much lower than had been reported by the manufacturers (Shah *et al*., 2000; Vinderola *et al*., 2000; Huff, 2004; Oliviera *et al*., 2007). There has been a recent upheaval in the public interest in probiotic cultures and the benefits they offer as part of a new 'healthy living' trend (MacFarlane and Cummings, 1999; Sanders, 2000; Lutter and Dewey, 2003)

There are many factors that decrease the numbers of live probiotic organisms in the human body. The two main factors are the low pH environment in the stomach and the presence of bile in the duodenum. Several studies have shown that bifidobacteria are sensitive to these environments (Lankaputhra and Shah, 1995; Brady *et al*., 2000; Ouwenhand *et al*., 2000). This limits the use of probiotics in the industry as many of the cells die during gastrointestinal transit (Hansen, 2002; Argawal, 2005).

This problem could be improved by microencapsulation of the probiotic organisms which could extend the shelf life. Microencapsulation as a tool for the protection of probiotic cells from the harsh gastric and intestinal environments have been previously attempted with differing levels of success (Sultana *et al*., 2000; Hansen *et al*., 2002, Krasaekoopt *et al*., 2004; Ding and Shah, 2007; Liserre *et al*., 2007; Kim *et al*., 2008). Most studies showed increased survival of the microencapsulated cells as opposed to the non-encapsulated cells (Khalil and Mansour, 1998; O'Riordan, 2001; Hansen *et al*, 2002; Critenden *et al*., 2006; Weinbreck *et al*., 2010). In certain studies enteric polymers designed for controlled release applications were used to microencapsulate cells. These polymers showed impressive protective effects under gastric conditions (Graff *et al*, 2008). The substances, however suitable for medical applications, are not permitted in food products (O'Riordan *et al*., 2001).

There is thus potential for microencapsulation of probiotic cells as a means of improving shelf life and as a protective measure against gastrointestinal conditions, especially gastric acidity. The testing of $scCO_2$-mediated microparticles in an in vitro gastrointestinal environment and during storage has only been reported by Moolman *et al*. (2006), Thantsha (2007) and Mamvura (2012). The results show increased survival of encapsulated cells compared with non-encapsulated cells, but only with the use of certain polymers. The lack of information regarding the protective effect of $scCO_2$-mediated lipid-based microcapsules against *B. lactis* during storage and gastrointestinal environments warrants further testing. The use of lipid-based microcapsules has not been well explored (Heidebach *et al*., 2012). This study aims to determine the survival of Vegetal BM 297 ATO encapsulated *B. lactis* during storage and in simulated gastric and intestinal environments.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial cultures and excipient

Bifidobacterium lactis Bb12 was obtained in freeze dried form from CHR-Hansen. The culture was stored at 4˚C. Biogapress Vegetal BM 297 ATO (Gatefosse, France) was obtained in powder form and stored at 4˚C.

3.3.2 Encapsulation of bacteria

Freeze-dried *B. lactis* Bb12 and Vegetal BM 297 ATO were mixed in a 3:7 ratio (g bacteria: g excipient). Encapsulation was accomplished using a Particles from Gas Saturated Solutions (PGSS) reactor (Separex Equipments, France). All equipment was wiped with 70% alcohol and allowed to dry before contact with materials. The encapsulation formulation was then mixed and immediately transferred to a pre-heated 1 liter reaction chamber. The chamber was sealed, flushed and pressurized with sterile filtered CO_2 (99.995 % purity, Air Products) up to a pressure of 300 bar, with the temperature being adjusted to 50°C. The material was left to equilibrate for 3 h with intermittent stirring (200 rpm) activated after 1 h. Once the CO_2 was released from the reactor, the encapsulated product was collected directly from the reaction chamber.

3.3.3 Determination of total encapsulated bacteria

One gram of Vegetal BM 297 ATO microparticles was suspended in 9 ml of Ringers solution in a Greiner tube. The tube was vortexed for 30 s and incubated for 8 h at 37˚C. A 1 ml aliquot was serially diluted in Ringers solution up to 10^{-10} dilution and 100 µl of each dilution was pour-plated onto De Man, Rogosa and Sharpe agar (Merck) supplemented with 0.05% cysteine hydrochloride. The plates were incubated anaerobicaly at 37˚C in anaerobic jars with Anaerocult A gaspaks and Anaerocult C test strips (Merck) for 72 h. Numbers were determined by taking the mean of triplicate plates that showed statistically relevant numbers of characteristic bifidobacterial colonies. The experiment was repeated three times.

3.3.4 Storage of Samples

The non-encapsulated and encapsulated bacteria were stored in sterile glass bottles at either room temperature or 4˚C for 12 weeks. Subsamples (1 g) were removed from each bottle weekly to determine viable counts. The samples were not stored anaerobically to simulate the conditions under which the products will be stored by the consumers.

3.3.5 Preparation of simulated gastric fluid (SGF)

The method was done according to Lian *et al.* (2003) with minor modifications. Pepsin (3 g/L) was suspended in saline (0.5% w/v). The pH was then adjusted to 2.0 with a 1 M HCl solution using a Thermo Orion 410 pH meter. The solution was then filtered through a 45 µm filter membrane (Millipore).

3.3.6 Preparation of simulated intestinal fluid (SIF)

The method was done according to Lian *et al.* (2003) with minor modifications. Briefly, 1.67 g of monobasic potassium phosphate (Merck) was dissolved in 6.25 ml distilled water and vortexed for 15 s. Then 1.925 ml of 0.2 M NaOH was added followed by addition of 12.5 ml of distilled H_2O. The solution was mixed by vortexing for 30 s. Thereafter 0.25 g of porcine pancreatin (Sigma-Aldrich) was added and mixed. The pH of the solution was adjusted to 6.8 with 0.2 M NaOH using a Thermo Orion 410 pH meter.

3.3.7 Survival of bacteria in simulated gastric fluid (SGF)

One gram of the non-encapsulated or encapsulated *B. lactis* was added to 9 ml SGF respectively in Greiner tubes and vortexed for 30 s for cell dispersion. Samples were taken to determine initial viability of the bacteria. The Greiner tubes were then incubated anaerobically at 37˚C in a shaking incubator (50 rpm) for 2 h. One milliliter aliquots were taken at 0.5, 1 and 2 h to determine viable counts.

3.3.8 Survival of bacteria in simulated intestinal fluid (SIF)

For the non-encapsulated and encapsulated samples, the cells were harvested from the SGF suspension and the pellet was resuspended in 9 ml of SIF and then incubated anaerobically at

37˚C in a shaking incubator (50 rpm) for 6 h. One milliliter subsamples were then taken from the tubes at 2, 4 and 6 h to determine viable counts.

3.3.9 Enumeration of Bifidobacteria

Serial dilutions of 1 ml aliqouts were prepared in Ringer's solution up to 10^{-10}. One hundred microlilters of each dilution was pour-plated in triplicate using De Man, Rogosa and Sharpe agar (Merck) supplemented with 0.05% cysteine hydrochloride. The plates were incubated anaerobicly at 37˚C in anaerobic jars with Anaerocult A gaspaks and Anaerocult C test strips (Merck) for 72 h. The mean numbers of characteristic bifidobacterial colonies of 3 experiments, each with triplicate plate counts were performed.

3.4 RESULTS AND DISCUSSION

3.4.1 Survival during storage

Viable numbers of unencapsulated bacteria were higher than the encapsulated cells throughout storage at 4˚C. The numbers of unencapsulated cells decreased by 3.5 $\log_{10}$ cycles over 12 weeks, from initial levels of 1.3 x 10^{10} cfu/g to 3.2 x 10^{7} cfu/g. The encapsulated cells on the other hand showed a 5 $\log_{10}$ cycle reduction during the same period from initial levels of 5.6 x 10^{9} cfu/g to 1.6 x 10^{4} cfu/g (Fig 3.1). The encapsulated cells were present in a lower initial number. This could be attributed to the 1-2 $\log_{10}$ cycle reduction in numbers of viable cells caused by the encapsulation process (Chapter 2). The numbers of encapsulated viable cells dropped to below recommended levels of 1 x 10^{6-7} cfu/g (Fasoli *et al*., 2003) after 8 weeks. The unencapsulated cells were still present in sufficient amounts at the end of the 12 weeks. These results indicate that at refrigerated temperatures, the Vegetal BM 297 ATO microparticles did not confer any increase in shelf life as the encapsulated cells underwent an additional 1.5 $\log_{10}$ cycle reduction compared to their non-encapsulated counterparts. The oxidation of the lipid matrix could have lead to exposure of the probiotic cells to oxygen. This is thought to be the main reason for the reduction in number of viable cells (Simpson *et al*., 2005).

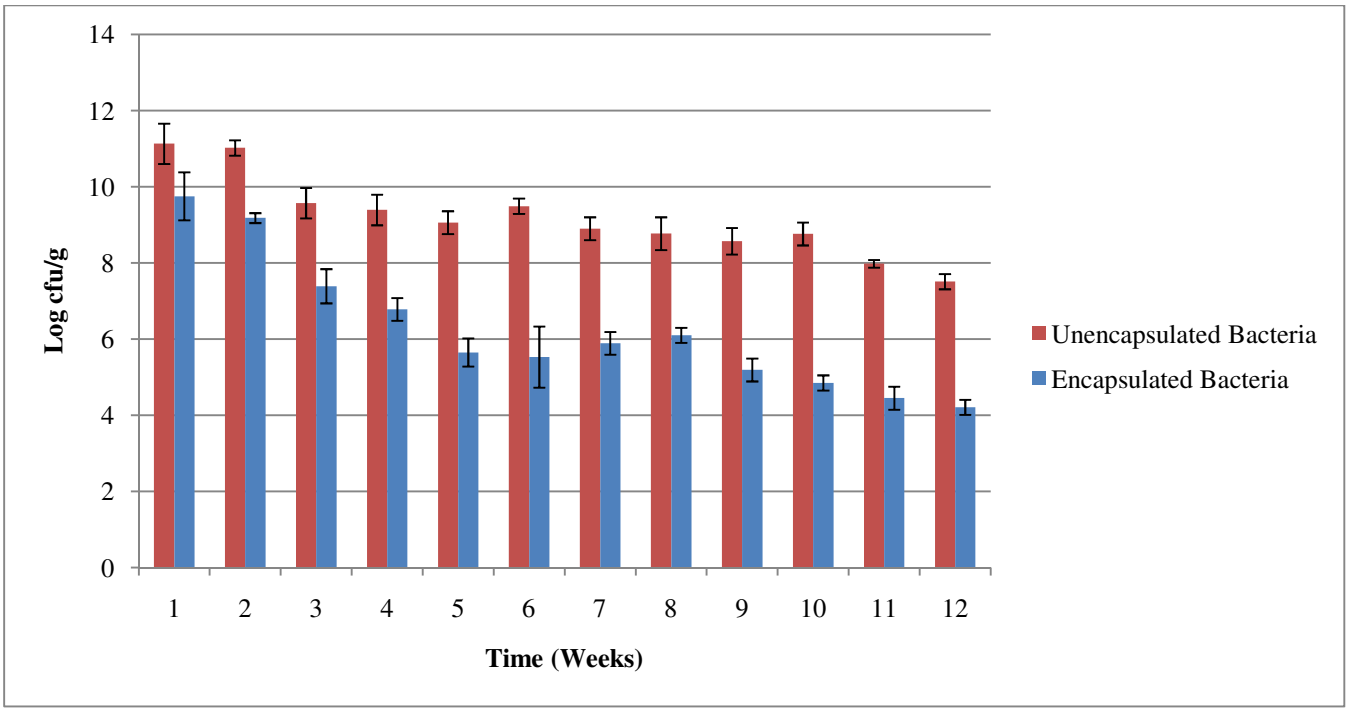

Figure 3.1 Viable levels of unencapsulated and Vegetal BM 297 ATO encapsulated *B. lactis* Bb12 during 12 weeks of storage at 4˚C. Each point represents the average of three replicate counts and error bars represent the standard deviation of three replicates.

A different trend was observed for samples stored at room temperature. The non-encapsulated cells decreased from initial levels of $8.1x10^{10}$ to 0 in three weeks while the encapsulated cells decreased from initial levels of $3.1x10^{9}$ cfu/g to 0 in the same period (Fig 3.2). Although the encapsulated cells were present in higher numbers than the unencapsulated cells at 21 days, their viability decreased to 0 cfu/g by day 28. The sudden decrease of viable numbers of cells at room temperature is in agreement with the findings of Simpson *et al.* (2005) who reported rapid decreases in certain species of bifidobacteria during different storage conditions. Some strains were found to be resistant to heat and oxygen, while 12 species were found to be sensitive to heat and oxygen. *B. lactis* was grouped among the species resistant to these factors. Oxygen stress and humidity are thought to be the main factors contributing to the rapid decrease of cells during storage at room temperature. Samples were stored in glass bottles as glass, as opposed to plastic bottles have been proven to increase the shelf life of probiotics due to their decreased permeability to oxygen (Shah, 2000, Hsiao *et al.,* 2004). As mentioned above, in this study resistance to oxygen permeability could have been compromised by re-opening of the bottles

during sampling as the subsamples were not stored separately. This is however very unlikely as the cells were not in the growth phase and so the effect of oxygen would be negligible.

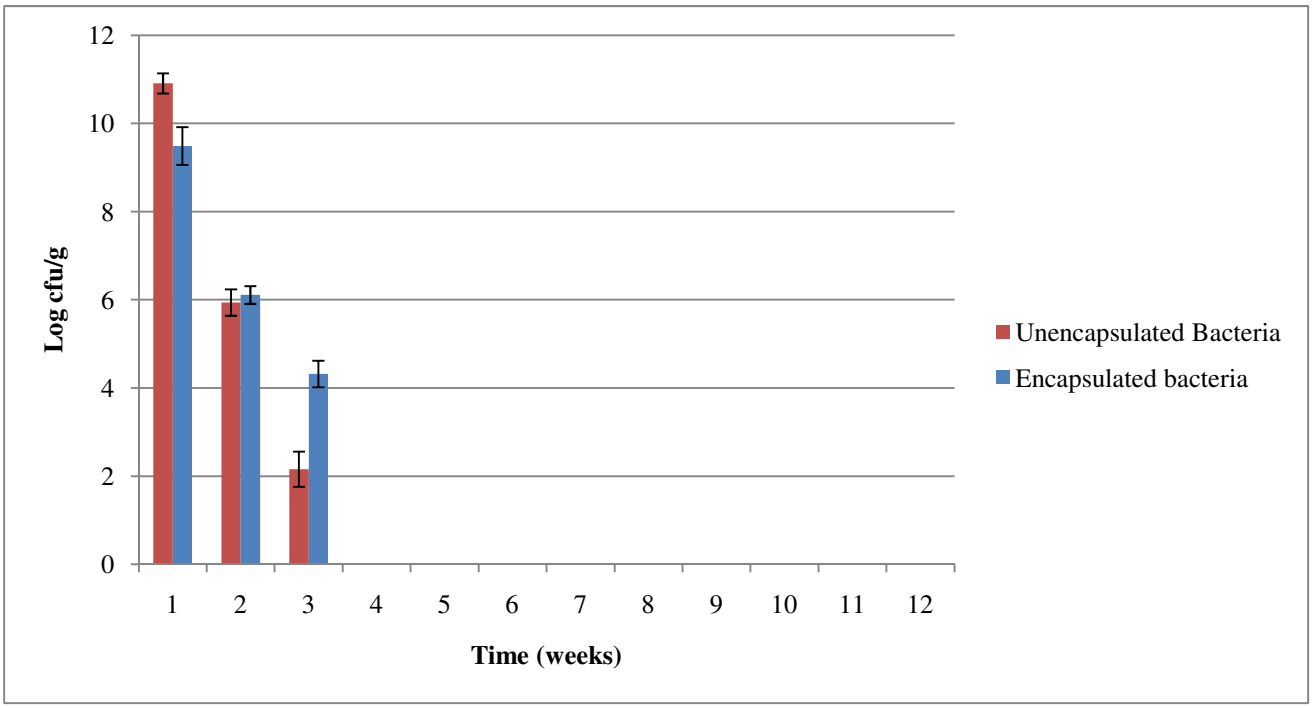

Figure 3.2 Viable numbers of non-encapsulated and Vegetal BM 297 ATO encapsulated cells during storage at room temperature over 12 weeks. Each data point represents an average of three replicate counts and the error bars represent standard deviation of three replicates.

This is the first study reporting the effect of lipid-based microcapsules formed by $scCO_2$ plasticization on the viable numbers of *B. lactis* during different storage conditions. Studies using a similar $scCO_2$ plasticization method were reported by Thantsha (2007) and Mamvura (2012), but utilized a pharmaceutical grade interpolymer complex as excipient. In the majority of the studies reporting the effect of microencapsulation on the shelf life of probiotics, microencapsulation was achieved using different methods. Most reported an increase in shelf life due to encapsulation. The results from this experiment are similar to a study by Thantsha (2007) which reported that encapsulated cells were present in lower numbers than non-encapsulated cells. The results show that microencapsulation using the PGSS method with Vegetal BM 297

ATO did not increase the survival of probiotic cells under aerobic storage. A study by Mamvura (2012) reported high levels of *B. lactis* present in samples after 12 weeks of storage. It was calculated that the encapsulation process increased the shelf life of the probiotic cultures by 7 weeks.

In this experiment the encapsulation had no effect on the shelf life of bacteria as the non-encapsulated cells decreased by roughly the same amount (3-4 $\log_{10}$ cycles, Fig 3.1). The encapsulated cells were present in lower initial levels, but showed a similar decrease as the non-encapsulated cells. O'Riordan *et al.* (2001) also reported no protective effect against bifidobacterium sp. when encapsulated in starch. However, other studies using different encapsulation methods reported improved shelf life of probiotic species during storage due to encapsulation (Critenden *et al*., 2006; Oliviera *et al.*, 2007, Weinbreck *et al*., 2010).

Alginate is the most frequently used excipient and is described as gentle towards the probiotic cells in microencapsulation due to its non-toxic nature and the easy release of cells from the matrix by use of a Ca^{+} sequestrating solution containing phosphate (Krasaekoopt *et al*., 2003). Alginate has been linked with enhanced cross-linking ability when there is a high percentage of glucoronic-acid (Orive *et al.*, 2006) but the bead sizes produced are large in comparison and thus present problems in food applications (Heidebach *et al*., 2012). The storage time for alginate-based microcapsules in food products range from 1-8 weeks (Hussein and Kebary, 1999; Sultana *et al*., 2000; Kailasapathy, 2006). Mamvura (2012) reported that encapsulation with an interpolymer complex of poly-(vinylpyrrollidone)-poly-(vinylacetate-co-crotonic acid) using a similar PGSS method with $scCO_2$ as a solvent did not offer significant protection to *B. lactis* Bb12.

As this is the first study to use a lipid-based excipient for microencapslation of probiotics using the novel encapsulation method no direct comparison to other studies can be made. The results should therefore serve as a benchmark in the evaluation of new excipients for suitability in microencapsulation for food applications using the PGSS method. The results indicate that Vegetal BM 297 ATO encapsulated *B. lactis* Bb12 should be stored under refrigerated conditions for optimal shelf-life.

3.4.2 Survival during simulated gastrointestinal transit

The unencapsulated and encapsulated bacteria were incubated in SGF for 2 h at 37°C as the emptying of one half of stomach contents is reported to be 1.5 h (Sun and Griffiths, 2000). The viable numbers of unencapsulated cells did not decrease in the gastric environment (Fig. 3.3). This is in contrast to Hansen *et al.* (2002) which reported a drop of 3-4 log cycles of *B. longum* at the same pH and temperature. The results however agree with Lian *et al.* (2003) which reported no significant decrease in the viable numbers of bifidobacteria at pH 2-3. This could be due to differences in activity of the membrane-bound ion transporters among different strains. The ability to regulate the proton-motive force is linked to increased acid tolerance (Booth, 1985). This is specifically linked to the activity of the H^+-ATPase transmembrane protein which can differ greatly from strain to strain (Matsumoto *et al*., 2004). The viable numbers of cells started to decrease at 2 h (Fig 3.3). The unencapsulated cells showed a decrease from initial levels of 1.2 x 10^5 cfu/ml to 1.1 x 10^4 cfu/ml after 6 h in the SIF.

The counts of the encapsulated cells decreased sharply during exposure to the gastric fluid but did recover to initial levels at 1 h (Fig 3.3). This suggests that the cells were not killed but rather temporarily inactivated. Exposure to the low pH could have induced a pH shock that decreased viable numbers as the cells prepared to survive in this new environment. The re-activation of cells could be due to cell-cell interactions as a result of exposure of cells near the surface of the microparticle matrix to a low pH as bifidobacterial strains are known to change luminal pH and secrete different metabolites in order to increase their own survival (Ohland and MacNoughton, 2010).

The viable numbers of *B. lactis* Bb12 released from the Vegetal microparticles increased over time during exposure to the SIF. This is thought to occur as the matrix dissolves and releases cells into the fluid. The numbers increased from an initial level of 9 x 10^3 cfu/ml to a level 1.4 x 10^6 cfu/ml after six hours of incubation (Fig 3.3). This shows a 1.5 log cycle increase in viable numbers. An increase in numbers above what was originally present suggests release of *B. lactis* Bb12 from the matrix as it dissolves and possible growth at pH 6.8.

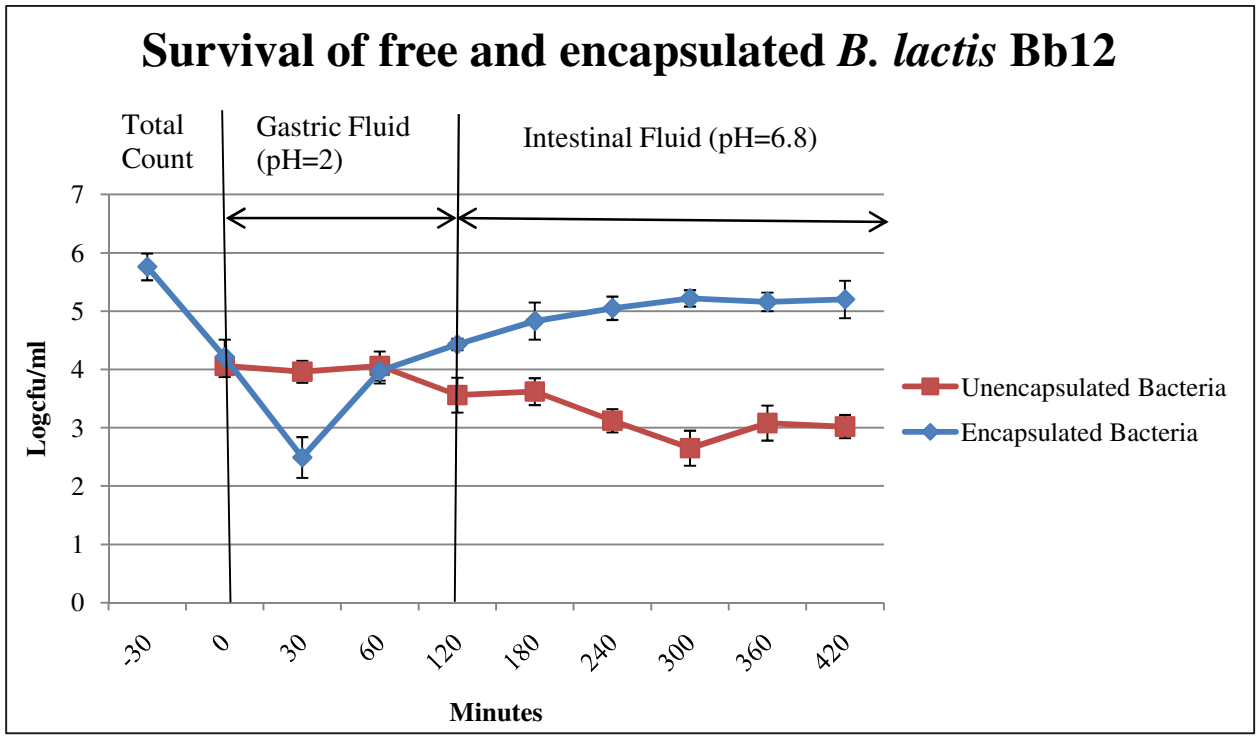

Figure 3.3 Survival of *B. lactis* Bb12 encapsulated in Vegetal BM 297 ATO during exposure to SGF and SIF over 8 h. Each data point represents an average of three replicate counts and the error bars represent standard deviation of three replicates.

The Vegetal microparticles resulted in increased survival of 2 log cycles during simulated *in vitro* gastrointestinal transit. This is similar to results obtained by Thantsha (2007) when poly-vinylpyrrolidone (PVP) along with beeswax was used to encapsulate *B. longum* Bb46 using $scCO_2$. The encapsulated cells showed a ~2 log cycle increased survival during simulated gastric transit. Similar results were obtained by Ding and Shah (2007) and Iyer and Kailasapathy (2005) using an alginate matrix, where a 2-3 log cycle protective effect was reported. The increased survival due to encapsulation by Vegetal was higher than other studies which reported no protective effect by different microcapsules on survival of different bifidobacterial and lactobacilli species during simulated gastrointestinal transit (Favaro Trindale and Grosso, 2000; Sultana *et al*., 2000; Hansen *et al*., 2002, Leverrier *et al*., 2005; Lisere *et al*., 2007). The Vegetal microparticles however showed less protection compared to reports by Cui *et al*. (2000), Lee *et al*. (2004) and Kim *et al*. (2008), which all reported a 4 log cycle protective effect. The Vegetal

microparticles are one of the very few lipid based microcapsules to show a protective effect during gastrointestinal transit. It outperformed many of its alginate-based counterparts. The capsules did not dissolve prematurely as a result of body temperature (37˚C) which was reported as a limiting factor for lipid based excipients by Picot and Lacroix (2004). These suggest that lipid based excipients can be just as suitable as the alginate based excipients in contradiction to Heidebach *et al.* (2012) which stated that lipid based excipients are currently limited in their applications in the food industry.

3.5 CONCLUSIONS

The results of the study showed that Vegetal BM 297 ATO microparticles created using supercritical carbon dioxide provides a protective effect to *B. lactis* Bb-12 during simulated gastrointestinal transit but do not contribute to shelf life. During gastrointestinal transit the novel lipid based microcapsules outperformed all but three similar studies using alginate-based excipient formulations and showed similar protective effects compared to interpolymer complex based microparticles created by a similar method. The results show that there is potential for lipid-based food grade excipients in the protection of probiotic cells during gastrointestinal transit and as a food additive, even though it does not improve shelf life.

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CHAPTER 4

GENERAL CONCLUSIONS AND RECOMMENDATIONS

4.1 GENERAL CONCLUSIONS

- Compritol E472 ATO is not suitable for use as an excipient using the PGSS process with supercritical carbon dioxide as a solvent for microencapsulation of *B. lactis* Bb12 due to high temperatures required for plasticization, which leads to unacceptable levels of cell death.

- Vegetal BM 297 ATO is suitable for use as a food grade excipient using the PGSS process with supercritical carbon dioxide as a solvent for microencapsulation of *B. lactis Bb12* as very little of the probiotic cells die during the process.

- *B. lactis* Bb12 was successfully encapsulated using Vegetal BM 297 ATO. This was due to a lack of probiotic cells on the surface of the microparticles using SEM analysis, and high numbers of probiotic cells on the inside of the microparticles using TEM analysis. There were no observable morphological changes to the probiotic cells as a result of the encapsulation procedure.

- The Vegetal BM 297 ATO microparticles containing *B. lactis* Bb12 were uniform in their irregular shape, had a mean particle size of 31µm and a smooth outer surface. A high bacterial load was observed inside the microparticles by releasing the cells from inside the particles and by the viewing of ultrathin cross-sections. The cells had an even distribution inside the lipid matrix. The particle size was desirable for use in food applications as it is small enough not to affect the texture and mouthfeel of the foodstuff to be added to.

- The Vegetal BM 297 ATO microparticles showed a high encapsulation efficiency of 88% which indicates that the PGSS process did not harm the cells. The process is thus effective and comparable if not superior to many of the currently used techniques.

- Microencapsulation by Vegetal BM 297 ATO using the PGSS process using supercritical carbon dioxide as a solvent did not extend the shelf life of *B. lactis* Bb12 at 4°C under aerobic conditions. The numbers of viable cells dropped below the recommended levels for use in food applications ($1x10^6$ cfu/g) after 8 weeks.

- Microencapsulation by Vegetal BM 297 ATO using the PGSS process using supercritical carbon dioxide as a solvent extended the shelf life of *B. lactis* Bb12 by 1 week during aerobic storage at room temperature. The samples showed no viable cells by the 4^{th} week. This indicates that Vegetal BM 297 ATO microparticles should be stored strictly at refrigerated temperatures. Oxidation of the lipid matrix was observed from the second week during storage at room temperature while the appearance of the powder stored at refrigerated temperatures remained unchanged by week 12.

- The Vegetal BM 297 ATO microparticles did impart gastrointestinal resistance as more viable cells were available in the sample containing encapsulated cells than the sample containing unencapsulated cells at the end of simulated in vitro gastrointestinal transit. Microencapsulation offered a ~$2\log_{10}$ cycle increased survival.

- The PGSS method using supercritical carbon dioxide was successfully used to microencapsulate *B. lactis* Bb12. The particles have desirable size, characteristics and impart gastric resistance. This technique can be applied to other food grade materials or sensitive bioactives and has potential for use in the food and pharmaceutical industries.

4.2 RECOMMENDATIONS FOR FUTURE WORK

- A new range of excipients need to be tested for suitability in microencapsulation applications using the method described in this study to create a database of suitable materials. Some materials may significantly increase shelf life as well as impart gastric resistance better than others. This will greatly broaden our knowledge and will create new opportunities for the sale and manufacture of these materials. The GRAS status of these materials negates the need for FDA approval and will lead to upstream cost savings in the microencapsulation process.

- The excipients need to be combined with additional bioactives such as prebiotics, essential oils and vitamins to see if multiple beneficial health-promoting agents can be incorporated into a food additive negating the need for them to be encapsulated and added separately. This will give the product a distinct market advantage and will lead to health benefits, as well as cost savings to the consumer.

- A larger range of reaction formulations need to be tested to determine the optimal use of materials for this method. This will indicate how to use materials most efficiently and therefore cost effectively.

- Different as well multiple strain formulations should be tested using this method to determine which strains are the best for use in microencapsulation of bioactives for use in food applications.