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LIST OF ABBREVIATIONS

AAD: Antibiotic Associated Diarrhoea WHO: World Health Organization

cfu: Colony forming units

CLSM: Confocal Laser Scanning Microscopy

DNA: Deoxyribonucleic acid

DVS: Direct vat set

FDA: Food and Drug Administration

FOS: Fructooligosaccharides

GI: Gastrointestinal tract

GMS: Glyceryl monostearate

GOS: Galactooligosaccharides

GRAS: Generally Regarded as Safe

HDL: High Density lipoprotein

IgA: Immunoglobulin A

IgE: Immunoglobulin E

LDL: Low Density lipoprotein

MRS: de Man Rogosa Sharpe

NDO: Non- digestible oligosaccharides

PEO-PPO-PEO: Ethylene oxide-propylene oxide triblock copolymer

PCL: Polycaprolactone

PGSS: Particles from Gas Saturated Solution

PI: Propidium iodide

PVP: Poly (vinyl pyrrolidone)

sASC: Specific Antibody Secreting Cells

$scCO_2$: Supercritical carbon dioxide

SCF: Supercritical Fluids

SEM: Scanning Electron Microscopy

SGF: Simulated Gastric Fluid

SIF: Simulated Intestinal Fluids

VA-CA: Poly (vinyl acetate-*co*-crotonic acid)

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SUMMARY

Incorporation of probiotic cultures in products in order to replenish or supplement the normal gastrointestinal microflora is a well known and accepted practice. However survival of these cultures is a problem due to a number of reasons including effects of storage conditions. Various researchers from different countries around the world have reported probiotic product instability. Microencapsulation has been used in an attempt to solve this problem. However, most methods involve the use of organic solvents which is not ideal because their toxicity may cause destruction of the microbial cells. A novel encapsulation method for probiotics, which excludes the use of organic solvents, was developed by the Council for Scientific and Industrial Research (CSIR) (US Patent Application no. 20050112205). This thesis investigated the efficiency/potential of this new method for increasing stability of sensitive probiotic cultures, specifically bifidobacteria.

Early studies using both culture dependent and culture independent techniques showed reduced numbers of viable cultures in probiotic products, mainly yoghurts, from all around the world. These results were confirmed in this study for similar products sold in South Africa. Most of the product labels did not specify viable numbers of probiotics nor the identity (genus and species names) of the microorganisms incorporated.

Successful encapsulation of bifidobacteria was achieved using the CSIR patented method. Complete encapsulation was indicated by absence of cells on surfaces of the encapsulated particles and production of a product with an acceptable particle size distribution was obtained. It was also demonstrated that the encapsulation process produced no visible morphological changes to the bacterial cells nor did it have a negative effect on cell viability over time. The potential of interpolymer complex formation in $scCO_2$ for the encapsulation of sensitive probiotic cultures was demonstrated for the first time.

Once ingested, probiotic cultures are exposed to unfavourable acidic conditions in the upper gastrointestinal tract. It is desired that these cultures be protected from this in order to increase the viability of the probiotics for efficient colonization. Interpolymer complex encapsulated *B. longum* Bb-46 cells were therefore exposed to simulated gastric fluid (SGF) and subsequently to simulated intestinal fluid (SIF).

It was found that the interpolymer complex protected bifidobacteria from gastric acidity, displaying pH-responsive release properties, with little to no release in SGF and substantial release in SIF. Thus the interpolymer complex demonstrated desirable characteristics retaining the encapsulated bacteria inside when conditions were unfavourable and only releasing them under favourable conditions. Survival was improved by the incorporation of glyceryl monostearate (GMS) in the matrix and by use of gelatine capsules. Protection efficiency of the interpolymer matrix was better when higher loading of GMS was used. Use of polycaprolactone (PCL) as an alternative to poly (vinylpyrrolidone) (PVP) and incorporation of ethylene oxide-propylene oxide triblock copolymer (PEO-PPO-PEO) affected the interpolymer complex negatively, rendering it swellable in the low pH environment exposing the bifidobacteria to gastric acidity. The use of beeswax seemed to have a more protective effect though results were inconclusive.

Probiotic cultures must also remain viable in products during storage. Encapsulated bacteria were either harvested from the reactor after 2 h of equilibration followed by depressurization, and then ground to a fine powder or after 2 h of equilibration the liquefied product was sprayed through a capillary tube with a heated nozzle at the end, into the product chamber. Encapsulated bacteria were stored in either sterile plastic bags or glass bottles under different conditions and then viable counts were determined over time. Survival of bacteria was generally better when the products were stored in glass bottles than in plastic bags. Bacteria encapsulated in an interpolymer complex formed between PVP and vinyl acetate-crotonic acid copolymer (VA-CA), (PVP:VA-CA) survived better than non-encapsulated bacteria under all storage conditions when the

product was recovered from the reaction chamber. When the product was recovered from the product chamber, numbers of viable non-encapsulated bacteria were higher than the encapsulated bacteria for all interpolymer complex formulations. This was probably due to some exposure to high shear during spraying into the product chamber. The interpolymer complex between PCL and VA-CA i.e. PCL:VA-CA seemed weaker than the PVP:VA-CA interpolymer complex as viable counts of bacteria released from it were lower than those from the latter complex. Addition of PEO-PPO-PEO to both the PVP:VA-CA and PCL:VA-CA complexes decreased the protection efficiency. However, results indicated that sufficient release of encapsulated bacteria from the interpolymer complexes was obtained when the encapsulated material was incubated in SIF rather than in Ringer's solution. When SIF was used for release of encapsulated bacteria, the shelf life of *B. longum* Bb-46 was doubled. Encapsulation in an interpolymer complex therefore provided protection for encapsulated cells and thus has potential for improving shelf life of probiotic cultures in products. Further studies will investigate the effects of encapsulating probiotics together with prebiotics in the interpolymer complex as well as effects of encapsulating combinations of different probiotic strains together, both on survival in simulated gastrointestinal tract and during storage.

The unique particles produced using the patented encapsulation technique increased the stability of probiotic cultures. This technique may find significant application in industries manufacturing probiotic products, especially food and pharmaceuticals, thereby improving the well being of consumers.

INTRODUCTION

There are twenty times more bacteria in the human body than cells. The large intestine alone contains about 10^{10}- 10^{11} bacteria g^{-1} of intestinal contents. This is made up of approximately 400-500 species, making the large intestine the most densely populated area in the whole body. These autochthonous bacteria have profound effects on the anatomical, physiological and immunological development of the host. Some members, good bacteria, are vital for good health while others, pathogens, are harmful and can cause infections. The good bacteria help promote digestion of food and absorption of nutrients. They also stimulate the host immune system to respond more quickly to pathogen challenge and inhibit colonization of the gastrointestinal (GI) tract by pathogens through bacterial antagonism. Pathogens produce chemicals toxic to the body and are frequently responsible for common digestive complaints such as constipation, diarrhoea and inflammation and for chronic conditions such as irritable bowel syndrome (Berg, 1996).

The GI tracts of healthy individuals maintain a balance between the good bacteria and pathogens. In these individuals intestines are colonized by favourable Gram positive microorganisms, notably lactobacilli and bifidobacteria (Fooks et al., 1999; Bin-Nun et al., 2005). When the balance between the two groups of bacteria is disturbed, the microflora population shifts towards prevalence of potentially detrimental microorganisms like *Clostridia*, sulphate reducing bacteria and *Bacteroides* (Fooks et al., 1999).

Factors contributing to imbalance include host physiology, microbial interactions (Richardson, 1996), lack of food or poor diet, travelling, antibiotics, cytostatics radiation, immune disorders, emotional stress and ageing (Havenaar and Huis in'tVeld, 1992; Richardson, 1996). Sites on the intestinal epithelium that were inhabited by beneficial microbes become empty. Occupation of these sites by potential pathogens increases the risk for outbreak of opportunistic infectious disease (Havenaar and Huis in'tVeld, 1992). Transient enteropathogens such as *Salmonella*, *Campylobacter*, *Escherichia coli* and

Listeria cause disorders such as cancer and ulcerative colitis. Susceptibility of the individual to infections is increased (Fooks et al., 1999) and other diseases e.g. liver and kidney disorders, atherosclerosis and hypertension may occur (Mitsuoka, 1996).

The normal balance of intestinal flora may be restored from an unbalanced state by deliberate ingestion of beneficial bacteria. Intestinal strains of lactic acid bacteria or bifidobacteria are used to fulfil this purpose (Mitsuoka, 1996). These strains of bacteria used to restore the balance of indigenous microflora of the gut are called probiotics.

The importance of autochthonous bacteria in the GI tract as a resistance factor against potential pathogens was already recognised in the 19th century by Metchnikoff. Research on probiotics started in 1950, although it was overshadowed and largely ignored due to introduction of antibiotics (Havenaar and Huis in'tVeld, 1992). Research in the field re-emerged in the 1960s due to the increased interest of people in health and natural ways of promoting health. The increase in numbers of bacteria acquiring resistance to multiple drugs, especially those causing nosocomial infections, the demand of consumers for natural substitutes for drugs, and the emergence of scientific and clinical evidence proving health benefits related to consumption of probiotic strains also made a contribution (Havenaar and Huis in'tVeld, 1992; Reid et al., 2003; Leahy et al., 2005).

Probiotics have been defined differently by various researchers, with the changes in the definition based on observations made when these particular microorganisms are studied. Fuller (1989) defined probiotics as "live microbial food supplements with health benefits to the host by improving the intestinal microbiota". Probiotics were later defined as "microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well being of the host" (Salminen et al., 1999). The World Health Organization and Food and Agriculture Organization of the United Nations (FAO/WHO, 2001) agreed on the definition of probiotics as: "live microorganisms which, when administered in adequate amounts confer a health benefit on the host" (Leahy et al., 2005).

The potential benefits of probiotic foods include: (Wahlqvist, 2002)

- Prevention and treatment of diarrhoea caused by rotavirus, especially in children
- Immune system enhancement
- Reducing some allergic reactions
- Treating and preventing respiratory infections, especially in children
- Decreased faecal mutagenicity
- Decrease in the level of pathogenic bacteria
- Decreased faecal bacterial enzyme activity
- Prevention of the recurrence of superficial bladder cancer
- The restoration of the correct balance of natural microflora after stress, antibiotic treatment, alcohol use and chemotherapy.

Today consumers are very cautious of their health and they expect the food that they consume to be healthy or even able to prevent illness (Mattila-Sandholm et al., 2002). Probiotics are available as tablets or capsules, powders, liquid suspensions and sprays (Marcon, 1997, Fooks et al., 1999). Some can be obtained from pharmacies as over-the-counter products (Marcon, 1997). In some countries like Japan, probiotics are incorporated into confectionery and fruit drinks. South Africa also has probiotic products on the market, comprising of different fermented milks and lyophilised preparations in the form of tablets or capsules (Theunissen and Witthuhn, 2004). The probiotic industry in South Africa is worth approximately R45 million per annum, with over 11 million doses taken annually. This means that over 30 000 doses of probiotics are taken daily in South Africa. The market for probiotics is developing and it is estimated to be growing at a rate between 8 and 15% for dietary supplements (Health 24, 2004).

The market for probiotics offers a great potential for manufacturers and is increasing although there are complex processing challenges of formulating products incorporating probiotics. The biggest challenge associated with the use of probiotics is the retention of viability of probiotic cultures during processing and storage. Probiotic products have to be efficient and reliable, i.e. they must contain sufficient numbers of viable microorganisms up to the expiry date (Fasoli et al., 2003). Their eventual success thus

depends on their survival in the products during their storage and their resistance to acidity in the upper GI tract, leading to establishment, colonization and ultimate efficiency (Sun and Griffiths, 2000: Picot and Lacroix, 2003). These bacteria often die during food manufacturing or during passage to the intestine. Shelf life has been unpredictable for probiotics, and the industry has had difficulty backing up label claims (Fasoli et al., 2003).

Probiotic bacteria perform best when they find suitable environmental conditions and when they are protected against stresses (e.g extreme temperatures, high pressure, shear forces) that they may encounter during their production at the industry level or in the gastrointestinal tract (gastric acids and bile salts) (Siuta-Cruce and Goulet, 2001). Harsh environments including exposure issues related to transport logistics, extended storage and the acidic conditions in the human stomach can kill live bacteria rendering probiotic supplements worthless by the time they are consumed or reach the intestines. This has been illustrated by numerous studies showing that most commercially available probiotic products do not deliver what they promise (Micanel et al., 1997; Vinderola et al., 2000; Huff, 2004).

This problem may be alleviated by use of probiotic encapsulation technology to ensure probiotic viability (Mattila-Sandholm, 2002). Development of delivery forms such as encapsulation techniques and coatings for protection of probiotics from detrimental factors leading to death is a significant area in probiotic research (Sun and Griffiths, 2000). Encapsulation of bifidobacteria for maintenance of viability has been investigated by various researchers (Hsiao et al., 2004). The most commonly used materials for immobilization of cells are alginate beads and κ-carrageenan (Sun and Griffiths, 2000). The encapsulation methods used typically employ organic solvents which are not favourable for use in this regard as solvents are generally toxic to microbial cells (Sardessai and Bhosle, 2002; Matsumoto et al., 2004). Probiotics are microbes and are therefore sensitive to these solvents. Solvents accumulate in the cytoplasmic membrane of cells changing its structure (Fernandes et al., 2003) and stopping the cell from

performing its normal functions (Kashket, 1987; Fernandes et al., 2003), which ultimately lead to cell lysis and death (Fernandes et al., 2003).

The technologies developed to produce gel beads present serious difficulties for large-scale production such as low production capacity and large bead diameters for the droplet extrusion methods and transfer from organic solvents and large size distribution for the emulsion techniques. Moreover, addition of some of the polysaccharides used is not permitted in yoghurts or fermented milk in some countries (Picot and Lacroix, 2004). Even though encapsulation of bifidobacteria for protection of viability has been investigated by various researchers, the methods and formulations still need to be refined.

Supercritical fluids have been widely used in extraction and recovery of high value compounds. A supercritical fluid is a substance that, at temperatures and pressures greater than its critical temperature and pressure, is a gas-like, compressible fluid that takes the shape of its container and fills it (Demirbaş, 2001). Experience accumulated in recent years on the use of supercritical fluids and their processes have indicated that it is possible to explore and envision their uses beyond the common practice of extraction (Sarrade et al., 2003). Supercritical fluid technologies can also be applied in making new innovative products. One of the very promising areas of research is microencapsulation of drug molecules, used for controlled drug release in the human body (Sihvonen et al., 1999). Supercritical fluids have the potential to contribute towards elimination of solvent toxicity problems as some of them (in particular carbon dioxide) can be used as a low temperature, stable, unreactive, environmentally benign solvent in encapsulation processes.

This work was part of a project entitled: “Supercritical fluid encapsulation of sensitive actives” where the main aim was to develop an encapsulation method using supercritical fluid, for protection and preservation of sensitive substances (like probiotics) in order to improve their viability, effectiveness and shelf life. If the method could overcome the problems posed by encapsulation methods using currently known technologies, it could benefit health care in South Africa in general, and particularly rural and remote areas of

the country. It could also be used for veterinary vaccines and other products if successfully developed.

The main objective of this research was to investigate the efficiency of the novel encapsulation technique for encapsulation of probiotics. The specific objectives were to:

- Determine viability of cultures in commercial South African probiotic yoghurts and to determine whether product labels specified the probiotic cultures and their levels in colony forming units (cfu) by the end of the shelf life**.**
- Investigate the efficiency of the novel method of probiotic encapsulation in interpolymer complexes and the effect of the encapsulation process on bacterial cells
- Determine the yield of probiotics that can be obtained after encapsulation into the polymer material.
- Determine whether encapsulation provides protection for probiotics in the gastrointestinal tract.
- Evaluate the effect of the encapsulation method on the shelf life of different probiotic microorganisms.
- Compare survival rates of the same *Bifidobacterium* strain when immobilised in different polymer complex formulations.
- Test the effect of incorporation of prebiotics on the stability (shelf life) of probiotics.
- Determine the effect of combining different probiotic strains on the survival rate.

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CHAPTER 1

LITERATURE REVIEW

1.1 Normal intestinal microflora

The indigenous microflora of the gastrointestinal (GI) tract in new-born humans and animals does not appear spontaneously. The foetus is sterile but it becomes contaminated with different microorganisms during birth. These microorganisms are then selected over time as changes occur in the GI tract. The different microorganisms inhabit different parts of the GI tract and then become characteristic of that particular habitat. Most of the microorganisms are thus eliminated such that at the end the GI tract of infants is dominated by lactic acid bacteria and coliforms. Further changes in the population of the microflora occur during weaning ending with the majority of the microbes being obligate anaerobes (Berg, 1996).

The microorganisms become distributed throughout the GI tract. Different parts of the GI tract become colonized by various populations of microorganisms. The stomach has a less dense microbial population which contains less than 10^3 cfu/ml of contents. Its population is dominated by aerobic Gram positive organisms. The small intestine separates the less populated stomach from the densely populated colon. The microflora of the small intestine is similar to that of the stomach but has higher numbers of microorganisms at the concentration of 10^3-10^4 cfu/ml (Berg, 1996; Richardson, 1996). The most densely populated part of the GI tract (and of the whole body) is the large intestine (colon) (Berg, 1996; Richardson, 1996; Taylor et al., 1999). In humans the intestinal contents contain about 10^{11} to 10^{12} bacteria per gram of stool. At least 500 species of anaerobic and facultative microbes, consisting among others several species of lactobacilli and bifidobacteria, are found in the contents (Percival, 1997; Wolfson, 1999; Losada and Olleros, 2002). Lactobacilli population comprising of about 60 species is found mainly in the small intestine while bifidobacteria are found in the colon. The *Lactobacillus* genus includes *L. acidophilus*, *L. planturum*, *L. casei* and *L. rhamnosus* while bifidobacteria include *B. longum*, *B. bifidum* and *B. infantis* (Wolfson, 1999). The microflora depends on dietary residues in the GI tract for their metabolism and has the potential to influence processes in the colon (Taylor et al., 1999).

The microorganisms in the intestines can be either beneficial or detrimental. This division is dependent on whether the various enzymes produced by these microorganisms perform functions that harm or support the host. The overall health of an individual depends on the balance between the beneficial and detrimental effects of the intestinal microflora (Percival, 1997). The major physiological functions of the gut microflora may be summarised as follows (Holzapfel and Schillinger, 2002):

- barrier function
- immune system stimulation
- maintenance of mucosa nutrition and circulation
- production of nutrient/improved bioavailability
- stimulation of bowel motility

The importance of indigenous microflora as a natural resistance factor against potential pathogenic microorganisms was originally recognised in the 19th century by Metchnikoff. However, research interest on the use of bacteria for therapy disappeared in the 1940's and then re-emerged around the 1960's due to increased interest of people in natural ways of promoting health. The demand for natural products such as fermented foods and bacterial cultures for alleviating diarrhoea has stimulated the industry to conduct research into new products (Havenaar and Huis int'Veld, 1992).

The indigenous microflora can be influenced by factors both in the internal and external environments, resulting in an imbalance between beneficial and detrimental microbes. External factors include lack of food and water, travelling, use of antibiotics, and drugs for treatment of tumours and radiation (Havenaar and Huis int'Veld, 1992; Luchansky et al., 1999). Other factors include peristalsis disorders, surgical operations of the stomach or small intestine, liver or kidney diseases, anaemia, immune disorders, emotional stress, poor diet and ageing ((Mitsuoka, 1996; Luchansky et al., 1999). Loss of indigenous microflora due to disturbances by one or more of these factors favour predominance of microflora by harmful bacteria ((Mitsuoka, 1996; Luchansky et al., 1999) and also result in availability of empty adhesion sites on the intestinal epithelium. These empty sites may be occupied by any organisms including transient pathogenic microorganisms.

Occupation of the empty adhesion sites by potentially pathogenic transient organisms may lead to an outbreak of an infectious disease (Havenaar and Huis int'Veld, 1992).

The balance between the detrimental and beneficial microflora may be restored to normal by a well balanced diet or by oral administration of bacteria. Strains of lactobacilli and bifidobacteria isolated from the intestines i.e. members of the indigenous microflora, can be administered to produce beneficial or health benefits (Mitsuoka, 1996). These bacteria prevent the growth of putrefactive flora through competitive inhibition, which can be by acidification of the medium, production of proteins with antibiotic activity and other hostile substances (Adams and Moss, 2000; Losada and Olleros, 2002). Their metabolic activities generate B vitamins, accompanied by proteolytic, lipolytic and β-galactosidase activity which improve tolerance to lactose (Adams and Moss, 2000; Losada and Olleros, 2002). They also improve digestion and hence absorption of different nutrients (Adams and Moss, 2000). Other benefits include improvement in disorders such as hepatic encephalopathy, stomatitis, vaginitis, intestinal infection, tumorigenesis and an increase in immune response (Losada and Olleros, 2002). These bacteria used for restoration of normal indigenous microflora are termed probiotics.

1. 2 Probiotics

Probiotics have been defined differently by various researchers. The definition has been modified with increasing knowledge in the field as researchers understand how probiotics function. Probiotics were defined as live microbial cultures fed by mouth and surviving transit through the large intestine where they colonise the system (Frost and Sullivan, 2000; Saarela et al., 2000; Matilla-Sandholm et al., 2002; Betoret et al., 2003). Schrezenmeir and de Vrese (2001) defined the term probiotic as a preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora by implantation or colonization, in a compartment of the host and by that, exert beneficial effects on host health. The World Health Organization and Food and Agriculture Organization of the United Nations (FAO/WHO, 2001) agreed on the

definition of probiotics as; “live microorganisms which, when administered in adequate amounts confer a health benefit on the host” (Leahy et al., 2005).

Probiotic food cultures have become popular due to appreciation of their contribution to good health (Desmond et al., 2002). In probiotic therapy, these beneficial microorganisms are ingested and thereby introduced to the intestinal microflora intentionally. This results in high numbers of beneficial bacteria to participate in competition for nutrients with and starving off harmful bacteria (Mombelli and Gismondo, 2000). The probiotics take part in a number of positive health promoting activities in human physiology (Chen and Yao, 2002).

The beneficial effects of the ingested probiotic bacteria are provided by those organisms that adhere to the intestinal epithelium (Salminen et al., 1998). The presence and adherence of probiotics to the mucous membrane of the intestines build up a strong natural biological barrier for many pathogenic bacteria (Chen and Yao, 2002). Adhesion is therefore regarded as the first step to colonization. Adhesion to the epithelium can be specific, involving adhesion of bacteria and receptor molecules on the epithelial cells, or non-specific, based on physicochemical factors. There are strains of lactic acid bacteria, both of human and dairy origin, which can effectively adhere to human cell lines (Salminen et al., 1998).

A specific microorganism has to fulfil a number of specific properties for it to be regarded as a probiotic strain. These properties are dependent on its specific purpose and on the location on which the specific property has to be expressed. The most important characteristic of probiotics include among others the following (Havenaar and Huis int’Veld, 1992):

- Survival in environmental conditions on the location where it must be active
- Proliferation and/or colonisation on the location where it is active
- No immune reaction against the probiotic strain i.e. the host must be immuno-tolerant to the probiotic

- No pathogenic, toxic, allergic, mutagenic or carcinogenic reaction by the probiotic strain itself, its fermentation products or its cell components after death of the bacteria
- Genetically stable, no plasmid transfer
- Easy and reproducible production

Lactobacilli were the first and largest group of microorganisms to be regarded as probiotics (Wolfson, 1999; Mombelli and Gismondo, 2000). They include *Lb. acidophilus*, *Lb. casei*, *Lb. delbrueckii* ssp. *bulgaricus*, *Lb. reuteri*, *Lb. brevis*, *Lb. cellobiosus*, *Lb. curvatus*, *Lb. fermentum*, and *Lb. planturum*, and Gram positive cocci such as *Lactococcus lactis* ssp. *cremoris*, *Streptococcus thermophilus*, *Enterococcus faecium*, *Streptococcus diacetylactis* and *Streptococcus intermedius*. (Krasaekoopt et al., 2003). Today probiotics include other lactic acid bacteria such as Bifidobacteria, Enterococci, *Leuconostoc* and Pediococci. Bifidobacteria occur naturally in the human intestines, with *Bifidobacterium infantis* being the first to inhabit the intestines of newborns. Other microbes used in probiotics but not related to lactic acid bacteria are yeasts (*Saccharomyces cereviciae*, *Saccharomyces boulardii*), filamentous fungi (*Aspergillus oryzae*) and some spore forming bacilli (Wolfson, 1999; Mombelli and Gismondo, 2000; Fuller, 2003)

Probiotic bacteria beneficially affect the individual by improving the properties of the indigenous microflora and its microintestinal balance (Frost and Sullivan, 2000; Saarela et al., 2000; Matilla-Sandholm et al., 2002; Betoret et al., 2003). They compete with disease causing bacteria for villi attachment sites and nutrients (Chen and Yao, 2002). Probiotic bacterial cultures encourage growth of beneficial microorganisms and crowd out potentially harmful bacteria thereby reinforcing the body's natural defence mechanisms (Saarela et al., 2000). They provide specific health benefits by modifying gut microflora, strengthening gut mucosal barrier, e.g. adherence of probiotics to the intestinal mucosa with capacity to prevent pathogen adherence, pathogen inactivation, modification of dietary proteins by intestinal microflora, modification of bacterial enzyme activity, and influence on gut mucosal permeability, and regulation of the immune system (Salminen et al., 1998; Betoret et al., 2003; Krasaekoopt et al., 2003).

Their probiotic effect is accredited to their production of metabolic by-products such as acid, hydrogen peroxide, bacteriocins, e.g. lactocidin, and acidophilin that manifest antibiotic properties and inhibit the growth of a wide spectrum of pathogens and/or potential pathogens such as *Escherichia coli*, *Klebsiella*, *Enterobacter*, *Pseudomonas*, *Salmonella*, *Serratia* and *Bacteroides* (Chen and Yao, 2002; Krasaekoopt et al., 2003). Lactic acid bacteria inhibit growth of pathogenic microorganisms by producing short chain fatty acids such as acetic, propionic, butyric as well as lactic and formic acids which reduces intestinal pH. Lactic acid produced by bifidobacteria in substantial amounts has antimicrobial activity against yeasts, moulds and bacteria (Percival, 1997; Adams and Moss, 2000). These species are also active in reducing the faecal activity of enzymes implicated in the production of genotoxic metabolites such as beta glucoronidase and glycolic acid hydroxilase (Collins and Hall, 1984; Mombelli and Gismondo, 2000). Probiotic organisms produce enzymes that help in digestion of proteins, fats and lactose (Frost and Sullivan, 2000). They also produce β-galactosidase, an enzyme that aid lactose intolerant individuals with breaking down or digestion of lactose (Krasaekoopt et al., 2003).

Commercially, viable probiotic strains are incorporated into fermented food products or are supplied as freeze-dried supplements or pharmaceutical preparations (Holzapfel and Schillinger, 2002). The basic requirement for probiotics is that products should contain sufficient numbers of microorganisms up to the expiry date (Fasoli et al., 2003). Thus, probiotics must contain specific strains and maintain certain numbers of live cells for them to produce health benefits in the host (Mattila-Sandholm et al., 2002). Different countries have decided on the minimum number of viable cells required in the probiotic product for it to be beneficial. In Australia, a minimum viable count of 10^6 organisms per gram should be available in fermented milk products at the end of the shelf life (Wahlqvist, 2002). However, according to Krasaekoopt et al. (2003), there are no specifications as to how many probiotics should be available in Australian fermented products. The same minimum count (10^6 organisms per gram) was approved by countries of MERCOSUR which includes Argentina, Paraguay, Brazil and Uruguay

(Krasaekoopt et al., 2003). In products containing multiple probiotic organisms, at least a million of each of them per gram should be present to produce required beneficial effects (Wahlqvist, 2002). In Japan, a minimum of 10^7 viable cells per millilitre of fresh dairy product is required. The South African legislation states that functional foods containing probiotic bacteria must deliver 1 x 10^8 bacterial cells per day. A daily intake of 10^9 to 10^{10} cfu viable cells is considered the minimum dose shown to have positive effects on host health (Fasoli et al., 2003). This could be achieved by consuming 100 g of a product containing between 10^6 and 10^7 viable cells g^{-1} daily (Boylston et al., 2004).

Retention of viability presents a major marketing and technological challenge for application of probiotic cultures in functional foods (Desmond et al., 2002; Mattila-Sandholm et al., 2002). Many active cultures die during manufacturing, storage or transport of the finished product (Siuta-Cruce and Goulet, 2001) and also during the passage to the intestine (Sakai et al., 1987; Siuta-Cruce and Goulet, 2001; Park et al., 2002). Thus, the majority die even before the consumer receives any of the health benefits (Siuta-Cruce and Goulet, 2001). A serious problem of shelf instability had been encountered with dried cultures. Refrigerated products also have short lives due to negative effects of low temperature and formation of crystals on bacterial cells. The numbers of viable bacteria continually decrease with time during refrigerated storage (Porubcan et al., 1975). Market surveys have revealed much lower counts in the products even before the expiry date (Talwalkar et al., 2001). Shelf life for probiotics is thus unpredictable; hence, the industry has had difficulty backing up label claims. (Siuta-Cruce and Goulet, 2001). Excesses of 50 to 200 % cells have been incorporated into products in an attempt to make-up for cells that die during storage. For example, in tablets containing dry cells, where the tablets are labelled as containing a certain minimum count of active cells per tablet, to be safe, the manufacturer must incorporate an excess of cells at the time the tablets are manufactured, thereby assuring that the labelling will remain accurate while the product is in stock by the retailers. This practice increases the cost and makes the use instructions inaccurate (Porubcan et al., 1975).

Probiotics, after surviving food processing, are then exposed to conditions prevailing in the stomach and small intestine before they reach their site which is the colon (Siuta-Cruce and Goulet, 2001; Hansen et al., 2002; Lian et al., 2002). The microbes may die during their transit through the upper intestinal tract to the colon and therefore they may not be able to colonize the colon (Talwakar et al., 2001). They must therefore survive gastric acidity and bile salts which they encounter during their passage through the GI tract (Sakai et al., 1987; Siuta-Cruce and Goulet, 2001; Hansen et al., 2002; Lian et al., 2002). Their survival in the GI tract depends on the strain and species-specific resistance to low pH (pH values ranging from 1.3 to 3.0) in gastric juice and to bile salts found in the small intestine (Hansen et al., 2002; Lian et al., 2002).

Probiotic bacteria can only perform when they find adequate environmental conditions and when they are protected against stresses (e.g extreme temperatures, high pressure, shear forces) they encounter during their production at the industry level or in the gastrointestinal tract (gastric acids and bile salts) (Siuta-Cruce and Goulet, 2001). Factors affecting viability during storage such as temperature, moisture, light and air should also be taken into consideration (Percival, 1997; Mattila-Sandholm et al., 2002). Oxygen toxicity is another major problem in the survival of probiotic bacteria in dairy foods. High levels of oxygen in the product are detrimental to the availability of these anaerobic bacteria (Talwakar et al., 2001).

Manufacturers of probiotics are facing the challenge that they should produce probiotic cultures that can survive for long periods in the products, and are resistant to acidity in the upper intestinal tract so that they can reach the colon in high numbers to colonize the epithelium. Probiotic cultures should therefore be produced in a way that will protect these sensitive bacteria from unfavourable interactions with detrimental factors (Siuta-Cruce and Goulet, 2001).

1.2.1 Bifidobacteria

Bifidobacteria are Gram positive, fermentative, strictly anaerobic rods, often Y-shaped or clubbed at the ends (Bergy, 1974; Mombelli and Gismondo, 2000). They are mostly of human origin (Mombelli and Gismondo, 2000). The most direct and definitive criterion for assigning bacterial strains to genus *Bifidobacterium* is to demonstrate presence of fructose-6-phosphate in cellular extracts (Berg, 1974; Orban and Patterson, 2000). Freshly isolated strains appear either as uniform rods or branched rods with Y and V forms and club or spatulate. The morphology of bifidobacteria cells is influenced by nutritional conditions. They are non-acid fast, non-spore forming and non-motile. They are saccharoclastic, i.e. they produce lactic and acetic acids without generation of CO_2 (gas is not produced during fermentation). Glucose is primarily fermented to acetic acid and L (+) lactic acid in the molar ratio of 3:2. Bifidobacteria are generally anaerobic but they differ in their tolerance to oxygen in the presence of CO_2. The G + C content vary from 57.2 to 64.5 % (Bergy, 1974).

Bifidobacteria have probiotic properties and therefore stimulating their levels in the colon may result in enhancement of immune system functions, improvement of digestion and absorption of essential nutrients and the synthesis of vitamins (Theuer and Cool, 1998). They are used in different conditions such as diarrhoea, for immune stimulation, as antimutagens and anticholesterol agents. Enzymes produced by bifidobacteria assist in the deconjugation of bile acid, catabolism of carbohydrates and synthesis of vitamins. They are used *in vivo*, especially in children, to restore the immune defence. They are mainly administered in combination in food (Mombelli and Gismondo, 2000). Their anaerobic nature however causes handling to be troublesome, resulting in their death during dehydration and plating (Fasoli et al., 2003).

Some of the most common *Bifidobacterium* species incorporated into probiotic products are discussed briefly below:

1.2.1.1 *Bifidobacterium bifidum*

They are Gram-positive rods highly variable in appearance. Under anaerobic conditions they form circular, convex or lens shaped colonies, whitish but not transparent, with smooth to mucoid soft surfaces. They give a final pH of 4.0 to 4.8 when grown anaerobically in glucose broth. *B. bifidum* does not hydrolyse gelatin, does not produce hydrogen sulphide and cannot produce ammonia from arginine. They require organic nitrogen to grow in the presence of fermentable carbohydrate. Their optimum temperature for growth is between 36-38 ^{o}C. They have variably limited growth at 23-25 ^{o}C and cannot grow at or below 20 ^{o}C or at 45 ^{o}C. Their optimum initial pH is between 6 and 7, with little or no growth at pH 5.5 or less. Strains of human and animal origin are non-pathogenic. They are found in the alimentary tracts and stools of breast fed infants and adults (Bergy, 1974).

B. bifidum is predominant in the intestinal tracts of breastfed infants though it is also present in adults. It was hypothesized that the reduced susceptibility of breastfed infants to infection when compared to bottle-fed infants, was due to the presence of *B. bifidum* in breastfed infants. *B. bifidum* inhibits the growth of competing pathogenic bacteria such as *E. coli*, *Shigella* and *Salmonella typhi*, through production of organic acids, leading to an increased hydrogen ion concentration in the growth medium. It also helps in the breakdown of complex carbohydrates, fat and proteins during digestion. They produce depolymerising enzymes that break the larger molecules down into smaller components that the body can utilise efficiently. These organisms have a detoxifying effect. For example, when *B. bifidum* was administered to 20 liver disease patients, a reduction in blood ammonia, free serum phenol and free amino nitrogen in the treated patients, was observed. The investigators attributed the positive effects to the *Bifidobacterium bifidum* bacteria (Nutraceutix, 2001).

1.2.1.2 *Bifidobacterium longum*

Bifidobacterium longum cells are long, curved, club shaped, swollen or dumb-bell shaped rods which may be bifurcated. Unlike *B. bifidum* which is Gram-positive, they are Gram-variable. They form convex to pulvinate colonies that are soft, moist, shiny or slimy. They ferment glucose to produce acetic acid and L(+)- lactic acid with no production of gas. They are also anaerobic, grow at 36-38 ^{o}C but not at 46.5 ^{o}C and 20 ^{o}C. *B. longum* has been isolated from faeces of infants and adults, and from the intestine of rats, guinea pigs and calves. It is present in adults throughout life (Boylston et al., 2004).

Beneficial effects of *B. longum* have been investigated in a number of clinical trials. It was indicated through studies that *B. longum* helps in digestion, due to its ability to degrade complex carbohydrates and improve levels of by-products of digestion such as ammonia and beta-glucuronidase. *B. longum* has also been used for prevention of antibiotic induced diarrhoea. Its antioxidative capabilities have been also shown in recent studies. *B. longum* cells and its cell extracts decreased levels of free radicals and increased inhibition of oxide cytotoxicity by 90 %. They chelate metal ions, especially copper, and scavenge reactive oxygen species such as hydrogen peroxide (Nutraceutix, 2001).

1.2.1.3 *Bifidobacterium adolescentis*

They are short, curved, occasionally bifurcated and anaerobic rods. They produce acetic and L(+)-lactic acid during glucose metabolism. They have an inducible gluconate fermentation pathway in which acid and gas (CO_2) are produced. They grow optimally at temperatures between 35-37 ^{o}C, with no growth at 46.5 ^{o}C and 20 ^{o}C. *B. adolescentis* have been isolated from faeces, appendix, dental carries and vagina of human adults and infants (Bergy, 1974).

1.2.1.4 *Bifidobacteriun infantis*

B. infantis cells are small, thin and spherical or bubble shaped, often containing central granules. Their growth conditions are similar to those of *B. bifidum* and *B. adolescentis*. They are predominant in the faeces of breast-fed infants (Bergy, 1974).

1.2.1.5 *Bifidobacterium breve*

Their cells are short, slender or thick, often club shaped rods, with or without bifurcation. Colonies are convex to pulvinate, smooth or undulating surface 2-3 cm in diameter, and have a soft consistency. They ferment glucose to produce acetic and L(+)-lactic acid. Gas is not produced during fermentation. They too, cannot grow at 46.5 ^{o}C and 20 ^{o}C. They have been isolated from infant faeces and from the vagina (Bergy, 1974).

1.3 Prebiotics

Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (Roberfroid, 1998; Theuer et al., 1998; Young, 1998; Femia et al., 2002). They change the intestinal microflora, favouring growth of potentially health-promoting bacteria, especially lactobacilli and bifidobacteria such that their numbers are predominant (Roberfroid, 1998). This subsequently lead to predominant numbers of stimulated endogenous bacteria in faeces as well (Femia et al., 2002; Losada and Olleros, 2002). They provide a beneficial effect through the selective stimulation of the growth or activity of a single species of bacteria which already resides in the colon, thereby improving a person's health. Prebiotics also modulate lipid metabolism through fermentation. The selective stimulation of growth of bifidobacteria by prebiotics is characterized by a substantial decrease in numbers of potentially pathogenic bacteria (Losada and Olleros, 2002).

Carbohydrates are used to promote growth of beneficial bacteria. These carbohydrates used for promotion of growth of lactic acid producing bacteria are called bifidogenic oligosaccharides (Farmer, 2002). Bifidogenic oligosaccharides include fructooligosaccharides (FOS) (inulin), glucooligosaccharides (GOS), other long chain oligosaccharides polymers of fructose and/or glucose and the trisaccharide, raffinose. They are not readily digested by pathogenic bacteria (Annika et al., 2002; Farmer, 2002; Femia et al., 2002).

Non-digestible carbohydrates are found in many fruits, vegetables (e.g. artichoke, garlic, leek and onion) and cereals (Femia et al., 2002; Losada and Olleros, 2002). They are a mixture of sugar chains formed by a glucose molecule and molecules of fructose joined together. Taking foods containing prebiotic oligosaccharides is not enough for modulation of gut flora as they are present in only small concentrations in these foods. Instead, prebiotics are extracted from these foods and transferred into more commonly ingested foodstuffs like biscuits and other carbohydrate based materials (Taylor et al., 1999). These natural compounds can also be manufactured economically using the β-D-fructofuranosidase enzyme or with fructosyltransferase, which joins the additional fructose molecules by means of transfructosylation mechanisms. Enzymes such as α-amylase, saccharase and maltase do not digest these oligosaccharides, especially in humans (Losada and Olleros, 2002).

This material reaches the colon, where it may be fermented completely or partially, or remain unfermented. None of the molecules of fructose and glucose that form inulin and oligofructose appear in portal blood. These materials are quantitatively fermented by colonic microflora. Fermentation of prebiotics by colonic microflora leads to the selective stimulation of the growth of bifidobacteria population (Flamm et al., 2001). In the GI tract bifidogenic oligosaccharides are metabolized exclusively by the indigenous bifidobacteria and lactobacillus and not by detrimental microorganisms such as *Clostridia*, *Staphylococcus*, *Salmonella* and *Escherichia coli.* The use of bifidogenic oligosaccharides together with lactic acid bacteria allows these beneficial, probiotic bacteria to grow and then out-compete any undesirable, pathogenic microorganisms

within the GI tract. Bifidogenic oligosaccharides increase the level of nutrient supplementation and enhance nutrient solubility (Farmer, 2002). Prebiotics unlike probiotics, are not living organisms, and therefore they do not have survival problems both in the products and the gut (Frost and Sullivan, 2000).

1. 3.1 Non digestible oligosaccharides (NDO's)

Oligosaccharides are available on the market as crystalline powders or 75% (w/v) syrups. The choice of oligosaccharides is affected by several factors such as regulatory considerations, the effects on the food's physiochemical properties and the stability of the oligosaccharide at differing pH and temperatures which may lead to hydrolysis of the NDO. Other factors that to be taken into consideration are the effects of the NDO on the human physiology, health claims that can be made for different NDO's as well as the cost of the NDO. NDOs have a number of physiological effects in humans. They are used as fat replacers because they are low in calories. They have low cariogenicity preventing erosion of teeth and bone, and they act as a form of dietary fibre. However their excess levels can cause symptoms such as flatulence, bloating and diarrhoea. This may be caused by a change in osmotic potential or due to excessive fermentation. Undesirable effects only occur when very high doses of NDOs are ingested. This is advantageous as it allows a relatively broad "therapeutic window", i.e. the dose above the minimal effective level (Holzapfel and Schillinger, 2002).

1. 3.2 Fructooligosaccharides(FOS)

FOS are oligosaccharides composed of a molecule of glucose and one to three molecules of fructose. They are polymers of β-D-fructosyl units having short length chains with a degree of polymerisation of up to 9 (oligofructose) to medium length chains with a degree of polymerisation of up to 60 (inulin). They occur naturally in many kinds of plants such as onions, asparagus roots, tubers of Jerusalem artichoke and wheat, but also in banana, beer, burdock, Chinese chives, garlic, graminae (fodder grass), honey, oat, pine, rye, chicory, stone leak and even bacteria and yeast (Ziemer and Gibson, 1998;

Bengmark et al., 2001). Short chain FOS are a mixture of oligosaccharides consisting of glucose linked to fructose units by β (1-2)- glycosidic bonds. They are not efficiently digested in the human small intestine but are fermented in the colon by colonic microflora (Bouhnik et al., 1999). Their monomers are joined by specific linkages that resist breakage by mammalian enzymes (Ziemer and Gibson, 1998). Most species of bifidobacteria are among the limited range of microorganisms able to ferment FOS. Bifidobacteria have relatively high activity of β-fructosidase that is selective for β(1-2)-glycosidic bonds present in these oligosaccharides. Fructose, a product of FOS hydrolysis, serves as an efficient growth substrate for the bifidobacteria pathway of hexose fermentation, which is almost exclusively carried out by bifidobacteria (Bouhnik et al., 1999). The presence of oligofructose and its fermentation products *in vitro* result in an increase in the number and metabolic activity of bifidobacteria (Theuer and Cool, 1998). Reports have indicated their beneficial effects on serum cholesterol and triglyceride levels, and blood pressure in elderly patients with hyperlipidemia (Bengmark et al., 2001).

FOS represents a selective nutrient for beneficial microorganisms and therefore has the potential to increase the effectiveness of current probiotic products. Scientific studies in Japan indicated that consumption of FOS shifts the balance of microflora in the intestine towards greater populations of bifidobacteria and other beneficial microorganisms even in the absence of probiotics in diet (Losada and Olleros, 2002).

They are produced commercially using two different manufacturing techniques that produce slightly different end products. During the first method FOS are enzymatically synthesised from sucrose using transfructosylases and in the second method inulin is derived from chicory. The polysaccharide is then subjected to a controlled enzyme hydrolysis (Frost and Sullivan, 2000). The two FOS produced are of similar structure and approximately equal size and hence there is a little difference in their prebiotic functionality. Studies involving humans and animals have established the effectiveness and safety of FOS. They have no genotoxic, carcinogenic or toxicological effects. FOS not only provide health benefits, they also contribute texture and body to the foods that

contain them (Frost and Sullivan, 2000). However, they are slightly laxative and produce flatulence when taken in high doses (Losada and Olleros, 2002).

1. 3.3 Galactooligosaccharides (GOS)

They are synthesised from lactose syrup using the enzyme β-galactosidase (Frost and Sullivan, 2000; Gibson, 2004). GOS are neither hydrolysed nor absorbed in the human intestine and act as a substrate for bifidobacteria (Frost and Sullivan, 2000).

1. 3.4 Soy oligosaccharide

They are extracted directly from soybean whey. Bifidogenecity of soy oligosaccharides has been confirmed in humans (Frost and Sullivan, 2000).

1. 3.5 Cereals

Cereal, nuts, legumes and oil seeds are sources of dietary fibre. Phytic acid is a hexaphosphorylated sugar and constitutes up to 1-5 % weight of these foods. Phytic acid chelates iron thereby inhibiting production of reactive oxygen species such as the hydroxyl radical. This may account for some of the protective effects of dietary fibre (Taylor et al., 1999). Cereals can also be used as fermentable substrates for the growth of probiotic organisms (Charalampopoulos et al., 2002). Fermentation of dietary fibre by colonic bacteria leads to the production of short chain fatty acids (Taylor et al., 1999; Ridlon et al., 2006).

Butyrate is one of the short chain fatty acids produced by fermentation of dietary fibre. *In vitro* tests and animal models have shown that butyrate increases the proliferation and differentiation of colonic cells. Apoptosis (programmed cell death) in human colonic tumour cell lines was also shown *in vitro* to be induced by butyrate (Taylor et al., 1999). Carcinogens such as nitrosamines are neutralized by butyric acid produced by some probiotic bacteria (Kailasapathy and Chin, 2000). The fermentation of fibre to short

chain fatty acids by colonic bacteria lowers the gut pH which may then reduce the conversion of primary bile acids to more toxic secondary bile acids (Taylor et al., 1999). Primary bile salts stimulate digestion and absorption of lipids and lipid-soluble vitamins in the intestines. However, when transformed to secondary bile salts they cause GI diseases such as colon cancer and gallstones (Ridlon et al., 2006).

Additionally, they can be used as sources of non-digestible carbohydrates that promote several beneficial physiological effects and also selectively stimulate the growth of lactobacilli and bifidobacteria present in the colon, thus acting as prebiotics. They contain water-soluble fibre, such as glucan and arabinoxylan, oligosaccharides, such as GOS and FOS and resistant starch, all of which have prebiotic properties. Starch can also be used as a coat for encapsulating probiotics in order to improve their stability during storage and enhance their viability during their passage through the adverse conditions of the GI tract (Charalampopoulos et al., 2002).

1. 4 Synbiotics

Synbiotics are products that contain both a probiotic and a prebiotic (Holzapfel and Schillinger, 2002; Touhy et al., 2003). They are defined as a mixture of a probiotic and a prebiotic that beneficially affects the host by improving the survival and establishment of live microbial dietary supplements in the GI tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health promoting bacteria and thus improving the host welfare (Kailasapathy and Chin, 2000; Touhy et al., 2003). Thus, synbiotics could improve the survival of the probiotic organism by providing specific substrate to the probiotic organism for its fermentation (Gallaher and Khil, 1999). Synbiotic supplements available include combinations of bifidobacteria and FOS, *Lactobacillus* GG and inulin and bifidobacteria, lactobacilli and FOS or inulin. Fermented milks contain both live beneficial bacteria (probiotics) and fermentation products that may positively stimulate the intestinal microflora (prebiotics). They are therefore referred to as synbiotics (http://www.invista.com/health/nutrition/biotics/synbiot.htm). Research has indicated the

potential of fermented milks in alleviating the risk of diseases such as colon cancer (Kieβling et al., 2002; Saikali et al., 2004). Synbiotics have antimicrobial, anticarcinogenic, immunomodulatory, antidiarrhoeal, antiallergenic, hypolipidemic, and hypoglycaemic activities. They may also have activity in improving mineral absorption and balance and may have antiosteoporotic activity (Gallaher and Khil, 1999; http://www.invista.com/health/nutrition/biotics/synbiot.htm).

1. 5 Application of probiotics in gastrointestinal dysfunctions associated with gut microflora imbalance

1. 5.1 Lactose indigestion

Lactose intolerance is the inability to hydrolyze lactose caused by lack of the enzyme β-galactosidase. This enzyme is found in large quantities at the tip of the villi of the small intestines (Salminen et al., 1998a; Vesa, 2000). It hydrolyzes lactose into glucose and galactose which are absorbed in the small intestine (Adams and Moss, 2000). Lactose intolerant individuals cannot digest a lot of lactose but only small amounts are metabolized by intestinal microbes (Salminen et al., 1998a). The undigested lactose passes to the colon where it is attacked by lactose fermenting organisms residing in the colon (Adams and Moss, 2000). Fermentation of lactose in the large intestine leads to production of hydrogen in breath (Mombelli and Gismondo, 2000). Incompletely absorbed lactose causes watery diarrhoea and large amounts of water may lead to dysfunctions of intestinal microflora (Salminen et al., 1998a).

Several strains of probiotics alleviate symptoms of lactose intolerance by providing lactase (β-galactosidase) to the intestine and stomach where lactose is degraded (Dairy Council of California, 2003). Probiotic strains produce β-galactosidase which breaks down lactose thereby improving tolerance to lactose (Fooks et al., 1999). Hydrogen in breath is used for diagnosis of lactose maldigestion (Vesa et al., 2000) or as an indicator of bacterial metabolism of lactose (Mombelli and Gismondo, 2000) whereby the amount of hydrogen in breath is equal to that of lactose not digested (Vesa et al., 2000). It has

been shown that hydrogen production is lower in subjects treated with fermented milk than in subjects with non-fermented milk. The low hydrogen content in those consuming fermented milk indicated that most of their lactose was metabolised (Mombelli and Gismondo, 2000).

1. 5.2 Constipation

Constipation is a disorder of motor activity of the large bowel characterized by bowel movements that are less frequent than normal (Salminen et al., 1998b). It is mainly caused by inappropriate diets, such as those low in fibre or non-starch polysaccharides. The symptoms of constipation are pain during defecation, abnormal swelling and incomplete emptying of colon contents (Salminen et al., 1998a). Total gut transit time in constipated individuals is generally longer than in non-constipated individuals, and stools are hard and difficult to pass (Salminen et al., 1998b). The link between intestinal microflora and constipation suggests that probiotics may be used for treatment and prevention of constipation.

In a study by Ouwenhand et al. (2002), twenty-eight constipated elderly subjects were enrolled in an open parallel study. The subjects were divided into three groups receiving juice, juice supplemented with *Lactobacillus reuteri* and the other supplemented with *Lactobacillus rhamnosus* and *Propionibacterium freudenreichii*. The results showed a 24 % increase in defecation frequency in subjects receiving the probiotic supplemented juice. Probiotics were then suggested to improve intestinal motility and reduce fecal enzyme activity.

1. 5.3 Antibiotic associated and rotaviral diarrhoea

Antibiotics cause diarrhoea in about 20 % of patients taking them. This is because antibiotics are non-selective, killing both pathogenic microbes and beneficial GI tract microflora (Tuohy et al., 2003). The antibiotic treatment shifts the equilibrium of the normal gut flora in favour of detrimental microorganisms (BergogneBérézin, 2000).

Reduction of beneficial microorganisms disturbs processes such as fermentation (BergogneBérézin, 2000) and compromises colonization resistance (BergogneBérézin, 2000; Tuohy et al., 2003), favouring growth of pathogens like *Clostridium difficile* and *Klebsiella oxytoca* (Tuohy et al., 2003). AAD results in poor digestion of carbohydrates, metabolized by colonic bacteria as an energy source. The normal production of lactic acid and short chain fatty acids (acetate, butyrate, and propionate) by anaerobic flora is decreased. Viable microorganisms (probiotics) can be used instead of conventional antibiotics to control GI disorders since they can restore the indigenous microflora (BergogneBérézin, 2000). Probiotics are seen as an important tool for improving health and nutrition of people in developing countries (Goldin, 1998).

AAD is usually caused by *Clostridium difficile*. Administration of probiotic *L. rhamnosus* GG either as powder or as a fermented product to children aged 4-45 months showed a significant reduction in duration of the diarrhoea. In a separate trial, administration of *Bifidobacterium* spp. and *Streptococcus salivarius* subsp. *thermophilus* as probiotic treatment resulted in reduction of the incidence of diarrhoea in the probiotic group after 17 months when compared to control group without treatment (Fuller, 2003). Recently, Kotowska et al. (2005) showed that *Saccharomyces boulardii* reduces the risk of AAD in children.

The common cause of diarrhoea in children is rotavirus infection. The intestinal mucosa is disturbed by infection, resulting in loss of microvilli and a decrease in the villus crypt ratio. Gut permeability increases, causing an increase in the absorption of macromolecules (Salminen et al., 1998a). Administration of *Lactobacillus rhamnosus* GG (LGG) resulted in significant reduction in the incidence of rotaviral diarrhoea compared with standard pasteurised yoghurt or placebo in paediatric populations. Additionally, a study of traveller's diarrhoea among 245 subjects who travelled to a developing nation for 1-3 weeks showed that the risk of developing diarrhoea on any given travel day was 7.4 % in the placebo group compared with 3.9 % in the LGG group. LGG provided a protection rate of 47 % against traveller's diarrhoea (Goldin, 1998; Gorbach, 2000).

Treatment and prevention of AAD by use of probiotics is one the most researched fields of probiotics in clinical applications and results thereof have been documented (BergogneBérézin, 2000; Marteau et al., 2001, McNaught and MacFie, 2001; Cremonini et al., 2002). The findings reported in these studies demonstrate that probiotics are effective in treating AAD in adults and rotaviral diarrhoeal disease in young children.

1. 5.4 Crohn's disease

Crohn's disease is an inflammatory disease of the GI tract occurring most commonly in the small intestine and the colon. It is caused by immunological disturbances, influence of agents such as bacteria or viruses that enter the lumen and activate the immune system, or inheritance. Crohn's disease is associated with destruction of the barrier function (Salminen et al., 1998a). Probiotics have been used in trials to treat this disease. *Lactobacillus* GG was administered orally for 10 d to sufferers of the disease. There was an increase in Immunoglobulin A (IgA) specific antibody secreting cells (sASC) to dietary B-lactoglobulin and casein. IgA is an antibody found in external secretions like saliva, tears, bile, urine and nasal, tracheobronchial, intestinal and cervical fluids. It is used to characterize the immunoglobulin patterns of these secretions. The potential of probiotic bacteria to increase gut IgA and thereby promote the gut immunological barrier has been indicated (Salminen et al., 1998b). IgA plays a very important function in local immunity and in creating a barrier against bacterial and viral infections (Fukushima et al., 1998).

1.5.5 Other application of probiotics

1.5.5.1 Food allergy

Food allergy is an immunologically mediated adverse reaction against dietary antigens. It is prevalent in infants because of the immaturity of their immune systems and the gastrointestinal barrier (Salminen et al., 1998a). An example of food allergy is that of infants with allergic reactions to milk due to casein. Some lactobacilli degrade casein into smaller peptides and amino acids. Studies showed that hydrolysis of different casein proteins by *Lactobacillus* GG decreased proliferation of mitogen induced human lymphocytes compared to non-treated caseins. Probiotics may exert a beneficial effect on allergic reaction by improving mucosal barrier function (Dairy Council of California, 2003). The supplementation of *Lactobacillus* GG to infants with atopic eczema after elimination of cow milk from their diet showed a significant reduction in the duration and intensity of atopic dermatitis. This may be due to reductions in intestinal inflammation and hypersensitivity reaction (Mombelli and Gismondo, 2000).

1.5.5.2 Atopic dermatitis

Atopic dermatitis is a common chronologically relapsing skin disorder affecting infants and children (Salminen et al., 1998a). It affects people who come from families with hay fever and asthma (hereditary). Individuals suffering from the disease develop rash on the skin and in addition have thickened itchy skin on the front of elbows, back of knees and on cheeks. Isolauri et al. (2000) investigated the effect of probiotics in infants who showed symptoms of atopic dermatitis during exclusive breastfeeding (i.e. no infant formula was given to these infants). The SCORAD score which measures the extent and severity of the disease was 16 in these infants. The infants were divided into groups and weaned to whey formula containing either *Bifidobacterium lactis* Bb-12 or *Lactobacillus* LGG. Skin conditions of infants receiving probiotic supplemented formula were improved when compared to infants receiving formula without probiotic cultures. The

SCORAD score was reduced to 0 in the bifidobacteria group and to 1 in the lactobacillus group.

The effect of *Lactobacillus* GG on atopic dermatitis was investigated in another study by Kalliomaki et al. (2001). *Lactobacillus* GG was given to expectant mothers whose relatives suffered from the disease and to their infants for 6 months after birth. Atopic eczema occurred twice in infants who together with their mothers received a placebo than in those receiving the probiotic.

Atopic dermatitis in pregnant and nursing mothers was prevented by consumption of probiotics. Children with this disease have high levels of allergic IgE antibodies. These levels were reduced in breast-fed infants whose mothers were fed *Lactobacillus*. Incidences of the diseases were fewer in these infants than in those whose mothers received a placebo. Atopic dermatitis occurred three times more commonly in the later group than in infants whose mothers received a probiotic (Mirkin, 2002). Reduction in the duration and severity of the disease may be due to both reduced intestinal inflammation and hypersensitivity reaction (Mombelli and Gismondo, 2000). The results of all the above mentioned studies indicated that probiotics may be used for the prevention of atopic disease.

1.5.5.3 Cholesterol and heart disease

Probiotics have the ability to lower levels of cholesterol in serum, contributing to the prevention of cardiovascular disease. In one study, men with high serum cholesterol levels were given a drink containing live lactobacilli and the control group was given a drink with no live lactobacilli. There was a 7.3 % reduction in total cholesterol and a 9.6 % fall in low-density lipoprotein (LDL) cholesterol in patients who received lactobacilli. No change in blood lipids, glucose or fibrinogen was observed in the control group (Proviva, 2002). LDL carries cholesterol to various tissues throughout the body. It is also referred to as “bad” cholesterol because high levels of LDL correlate most directly with coronary heart disease. Thus, the lower the levels of LDL in blood, the lower the

risk of heart disease or stroke. High density lipoprotein (HDL) carries excess cholesterol and probably other phospholipids and proteins, to the liver for "repackaging" or excretion in the bile. Higher levels of HDL are protective against coronary artery disease and as such HDL is referred to as "good" cholesterol.

Oral administration of *Lactobacillus johnsonii* and *L. reuterii* decreased serum cholesterol in pigs and rats (Mombelli and Gismondo, 2000). Probiotics probably interfere with cholesterol or produce metabolites that affect the system levels of blood lipids (Fooks et al., 1999). For example, reduction in cholesterol levels may be attributed to deconjugation of bile salts by hydrolase, an enzyme produced by some lactobacilli strains (Mombelli and Gismondo, 2000).

1.5.5.4 Cancer

Probiotics have powerful anticarcinogenic features that are active against certain tumors (Chen and Yao, 2002). Several studies indicated that probiotics in diet reduce the risk of cancer (Sanders, 1999). Clinical trials in humans showed anticarcinogenic effects of *Bifidobacteriun bifidum* and *Lactobacillus acidophilus* (Fooks, 1999). Production of short chain fatty acids in the colon during fermentation by colonic microflora is the main process that prevents colorectal cancer (Holzapfel and Schillinger, 2002). Probiotic strains also reduce levels of some colonic enzymes such as glucoronidase, β-glucoronidase nitroreductase, azoreductase (Fooks et al., 1999; Adams and Moss, 2000; Gorbach, 2000; Chen and Yao, 2002) and glycoholic acid hydrolase (Chen and Yao, 2002). These enzymes convert procarcinogens to carcinogens such as nitrosamine or secondary bile acids (Chen and Yao, 2002). Low levels of these enzymes therefore decrease chances of cancer development in the colon (Kasper, 1998; Gorbach, 2000).

A synbiotic containing *B. longum* and inulin was shown to reduce risks related to tumors. Animal models also indicated that lyophilised *B. longum* suppressed the development of azoxymethane induced aberrant cryptic foci formation in rat tumours. How exactly

probiotics produce antitumor action is unknown but a few possible mechanisms in addition to those mentioned above, were proposed (Fooks et al., 1999):

- reducing intestinal pH, thereby altering microflora activity
- altering colonic transit time to remove faecal mutagens more effectively
- stimulation of the immune system

1. 6 Shelf life stability of probiotics

A number of studies have indicated that probiotic cultures do not survive satisfactorily in most products. Nevertheless, some researchers and companies claim that their products are stable and have acceptable shelf lives. A few of these are described.

Universal Preservation Technologies have preserved probiotic cultures such as *Lactobacillus acidophilus*, *Lactococcus lactis* and *Bordetella*, at temperatures above 50 ^{o}C. Bacteria are preserved using specially developed fermentation and drying protocols and protectants in a process termed Vitrilife preservation. In this process bacteria are reported to be produced as a dry product that can be reconstituted with an aqueous solution. It is also reported that bacteria preserved using this process can survive at 37^{o}C for 30 d with no loss of viable cells (Universal Technologies, 2000).

Achour et al. (2001) preserved *Lactococcus* starter cultures by freeze-drying. Cells are harvested by centrifugation and then washed with a saline solution. The sample is supplemented with $CaCO_3$, then glycerol or 5% saccharose. The product has a half life of 7 d at 25 ^{o}C and 43 d at 4 ^{o}C. Worthington et al. (2001) claim a shelf life of 6 -12 months for flavoured fruit yoghurt. The product is stabilized by adjusting the pH to optimum for cultures used and then frozen in the presence of gas and then blended with a low moisture food. The frozen mixture is then extruded into a bar or other intended form, freeze-dried and then packaged.

Kafanani and Mize, (2002) produced a milk composition containing probiotics in amounts sufficient to benefit the consumer's health. The composition is ultrapasteurized, cooled to about 20-30 ^{o}C and inoculated with a probiotic culture that has been prepared under aseptic conditions. The resulting milk composition is ready to use, has an extended shelf life and contains sufficient probiotics (at least 10^{8} cfu/ml) to be beneficial to the consumer even after more than 90 days. The milk is filled into containers which are sealed with a sterile closure under aseptic conditions. The containers are flushed under aseptic conditions with sterile gas, typically nitrogen to remove oxygen from the container just before sealing.

Nutraceutix uses technology called cryotabletting. The process employs liquid nitrogen to reduce heat during tabletting, resulting in significantly less loss and a more potent tablet. The process involves every step of the manufacturing process, including careful strain selection, and step-by-step monitoring of the starter culture growth, fermentation, freeze-drying, blending, tabletting and bottling process. LiveBac ^{TM} process produces probiotic tablets that do not require refrigeration and that are stable at room temperature for more than a year. (Nutraceutix, 2002).

Though longer shelf lives have been claimed, surveys on probiotic products still reveal that stability of cultures is a problem. As such, researchers are using different approaches to develop methods that will successfully preserve viability of probiotic cultures.

1.7 Moving towards improving shelf life of probiotics

1.7.1 Cell immobilisation

Immobilization is the physical entrapment of microbial cells in or on a polymer matrix. Although immobilized cells are separated from the medium containing substrates and products, exchange of substrates, products and inhibitors between the two still occurs. The microenvironment within which the immobilized cells exist differs from that of free cells (Ramakrishna and Prakasham, 1999).

When cells are immobilized inside a matrix, the matrix provides protection for cells against harsh environmental conditions such as pH, temperature, organic solvents, water molecules and poison (Bryers, 1990; Park and Chang, 2000. Productivity of lactic acid bacteria during fermentation may be improved through this process. When cells are artificially immobilized they do not grow, and this is advantageous as it minimizes the chances of contamination normally associated with growing cells (Bryers, 1990).

Methods of cell immobilization include cross-linking, entrapment (Tanaka and Kawamoto, 1999, Ramakrishna and Prakasham, 1999), adsorption, covalent binding and encapsulation (Ramakrishna and Prakasham, 1999). These methods are classified based on the mode of attachment of cells which can be mechanical, chemical or ionic. Mechanically immobilized cells are localized by means of physical barriers, chemically immobilized cells by covalent bonds formed among cells or between cells and the solid phase. Electrostatic and van der Waals or London forces are used in ionic immobilization (Phillips and Poon, 1988). Each method has its own advantages and disadvantages.

1.7.1.1 Entrapment method

This is the most commonly used method for immobilization of cells. It involves incorporation of the cells within a network of a polymeric material such as carbohydrate, protein, and organic or inorganic synthetic polymers (Phillips and Poon, 1988). Cells are entrapped using cellulose, its derivatives, gel-like extractions from seaweeds such as agar, alginate and carrageenan, and pectin from skins of citrus fruits (Phillips and Poon, 1988; Picot and Lacroix, 2004). The entrapped cells remain stable as the polysaccharides used are similar to the physical environment found in microbial cells (Phillips and Poon, 1988).

Cellulose and its derivatives are insoluble in water, but dissolve in polar aprotic organic solvents such as dimethyl formamide, acetone, and dimethylsulphoxide. Therefore when microbial cells are entrapped using cellulose, they are first added to a solution containing

an aprotic solvent. The solution is then passed into water where it is drawn as fibres or formed into beads and membranes containing the immobilized microorganism. κ-carrageenan is used in conjunction with a hardening agent gluteraldehyde, giving high carrier stability and long microbial half-lives. κ-carrageenan provide favourable conditions for viable microorganisms. Polyacrylamide is the most commonly used synthetic polymer. It is soluble in water and is normally cross-linked to the co-polymer N, N'-methylenebisacrylamide to produce a polymer with lattice like structure better suited to cell immobilization. (Phillips and Poon, 1988).

However, this method has a number of disadvantages. Alginate beads are sensitive to acid and it was reported that they shrink and lose mechanical strength during lactic fermentation. The formation of κ- carrageenan beads requires potassium ions, which could damage bacterial cells during fermentation. Potassium ions are involved in the maintenance of electrolyte balance in body fluids and should therefore not be taken in large amounts in diet (Sun and Griffiths, 2000). Agar is unstable towards high temperature and calcium alginate is unstable in the presence of chelating agents such as phosphate salts (Phillips and Poon, 1988). The technologies for production of gel beads present serious problems for large-scale production such as low production capacity, large bead diameters for the droplet extrusion methods, large size distribution for the emulsion techniques and transfer from organic solvents. Also in some countries addition of these polysaccharides to fermented milks is not allowed (Picot and Lacroix, 2004).

1.7.1.2 Covalent attachment

In this method, covalent bonds are formed between cells and the polymer lattice, or among the cells themselves to form a mat. There is contact between cells and chemical reagents and this normally leads to death of cells (Phillips and Poon, 1988). Loss of viability of immobilized cells is a disadvantage especially when live cells are needed.

1.7.1.3 Ionic attachment

It involves formation of electrostatic forces such as hydrogen bonding, coordinate binding, Van der Waals and dispersion forces. Microbial cells can be immobilized by ionic attachment either by flocculation or adsorption. The most commonly method used is flocculation which is usually used in fixed bed reactors (Phillips and Poon, 1988).

1.7.2 Microencapsulation

Microencapsulation is a process whereby sensitive actives such as microbial cells are enclosed within a protective coat (Vasishtha, 2003). Encapsulation reduces cell loss by separating bacterial cells from the adverse environment (Sultana et al., 2000). The protective coat reduces cell loss and injury by blocking reactive components such as atmospheric moisture, oxygen and acids (Kim et al., 1988; Reid, 2002; Krasaekoopt et al., 2003; Vasishtha, 2003,). The coat also protects cells from high temperature and pressure, attack by bacteriophages, negative effects of freezing and freeze-drying (Krasaekoopt et al., 2003). The sensitive active or the core material can be retained within a coat until it is released at a particular targeted location (Finch, 1993; Vasishtha, 2003). Targeted release in case of probiotics can be obtained by use of coating materials made from sugars that do not allow hydration until the probiotics reach the alkaline pH of the colon (Reid, 2002; Krasaekoopt et al., 2003). Encapsulation allows cells to tolerate acidity better (Siuta-Cruise and Goulet, 2001). This process can increase shelf life of cultures by slowing down the rate of viability loss at room temperature (Kim et al., 1988) and of food products by alleviating problems encountered during processing (Finch, 1993; Vasishtha, 2003). Through this process, manufacturing of new products, protection of the environment from poisonous products and masking of unpleasant tastes of some nutrients, are made possible (Finch, 1993; Vasishtha, 2003).

It has been found that lactic acid bacteria enclosed within solid fat microcapsules retain all their activity or vitality (Krasaekoopt et al., 2003). The technique has been applied to strains of probiotics used in food applications including *Lactobacillus acidophilus*,

Lactobacillus rhamnosus and *Bifidobacterium longum*. *Pediococcus acidilactici* and *Enterococcus faecium* were incorporated in animal feeds (Siuta-Cruce and Goulet, 2001).

It is difficult to commercialize encapsulated product as only coats made from FDA-approved GRAS (generally recognized as safe) materials will be accepted. The GRAS shell material must stabilize the core material, must not react negatively with the active ingredient rendering it inactive, and should release at the target site. The production process must be able to produce a stable product with desired morphology, and large scale production must be cost effective (Vasishtha, 2003).

Different methods of encapsulation are classified either as physical or chemical (Versic, 1988; Vasishtha, 2003). Physical methods include use of commercially available equipment to create and stabilize the capsules (Vasishtha, 2003). Examples include spray coating, annular jet, spinning disk, spray cooling, spray drying and spray chilling (Versic, 1988), extrusion coating, fluidized bed coating, liposome entrapment, coarcervation, inclusion complexation, centrifugal extrusion and rotational suspension separation (Vasishtha, 2003). Chemical methods are water-in-oil and oil-in-water (complex coacervation) preparations (Versic, 1988). They apply ionic chemistry to create the microspheres in batch reactors (Vasishtha, 2003). Two methods of encapsulation commonly used are the extrusion technique and emulsion technique (Krasaekoopt et al., 2003). The release of the core from the coat can be either site specific, stage specific or signalled by changes in pH, temperature, irradiation or osmotic shock (Gibbs et al., 1999; Vasishtha, 2003).

1.7.2.1 Extrusion

In this technique, a hydrocolloid solution is prepared, microorganisms are added to it to form a suspension, and then the suspension is extruded as droplets through a syringe needle into a hardening solution or setting bath. The size of the beads depends on the diameter of the needle, and the concentration and viscosity of sodium alginate, while the shape depends on the distance of free fall from the needle into the hardening solution.

The supporting material commonly used is alginate, extracted from various species of algae. To form beads, a cell suspension is mixed with a sodium solution, the mixture dripped into a solution containing a multivalent cation. The multivalent cation used is usually Ca^{2+} in the form of $CaCl_2$. The droplets form gel spheres which entrap the cells in a network of ionically cross-linked alginate. The size of beads decreases with an increase in the concentration and viscosity of sodium alginate (Krasaekoopt et al., 2003).

1.7.2.2 Emulsion

In this technique a small volume of cell polymer suspension is mixed with a large volume of vegetable oil e.g. soybean oil, sunflower oil, canola oil or corn oil. The mixture is then homogenized to form a water-in-oil emulsion. Once the emulsion is formed, the water-soluble polymer is cross-linked to form tiny gel particles within the oil phase. A $CaCl_2$ solution is then added to the homogenized mixture to break the emulsion and form a gel. The beads are harvested by filtration. The size of beads is controlled by the speed of agitation, and can vary between 25 μm and 2 mm. This technique had been used to encapsulate lactic acid bacteria for both batch and continuous fermentation. A number of supporting materials can be used. They include a mixture of κ-carrageenan and locust bean gum, cellulose acetate phthalate, alginate, chitosan and gelatin (Krasaekoopt et al., 2003). The disadvantage of emulsion the technique is that it is difficult to produce large quantities of beads and to remove oil from them (Stormo and Crawford, 1992).

The advantages and disadvantages of extrusion and emulsion encapsulation techniques are tabulated in Table 1.1. (Krasaekoopt et al., 2003).

Table 1.1: Positive and negative features of extrusion and emulsion techniques

	Extrusion	Emulsion
Technological feasibility	Difficult to scale up	Easy to scale up
Cost	Low	High
Simplicity	High	Low
Survival of microorganisms	80 to 95%	80 to 95%
Size of beads	2 to 5 mm	25 µm to 2mm

The main disadvantage of these methods is use of water and other solvents. The sensitive active, specifically probiotics, need protection from moisture as it is unfavourable for their survival. Also, use of solvents is no longer favoured due to concerns with their impact on the environment, and due to their high costs (Sihvonen et al., 1999). Therefore, a method that will exclude use of water and solvents will be more favourable, both to the encapsulated active and the environment.

1.7.2.3 Spray drying

Spray drying is a process in which an aqueous solution containing the sensitive active core material and solution of the wall material are atomised into hot air (Finch, 1993; Reineccius, 1988). The process involves three basic steps: preparation of a dispersion or emulsion to be processed; homogenization of the dispersion; and atomization and introduction of the mass into the drying chamber under controlled temperature and inflow conditions (Niro Inc, 2004). Products of spray-drying can be in powder, granulate or agglomerate form. Heat sensitive foods and pharmaceuticals are among products dried using this method (Rattes and Oliveira, 2004).

Spray drying has a number of advantages. Equipment used is readily available, the process is relatively cheap, it is compatible with a variety of carrier materials, large quantities of microcapsules can be produced, volatile substances can be easily retained and its products are stable (Reineccius, 1988; Picot and Lacroix, 2004). Like many other methods, it has problems too. Its main disadvantage is loss of viability of cells. Reduction in cell viability after spray drying was suggested to be a result of shear by atomizing air pressure, heating inside the atomizer, dehydration and thermal inactivation. These problems limit the application of spray-drying specifically for encapsulation of sensitive probiotic bacteria such as *Bifidobacterium* spp. Reduction in viability can be lessened by lowering the outlet air temperature which is the main cause of cell death. However, this approach cannot be applied to small spray dryers which are unable to achieve complete and satisfactory drying of suspensions at outlet air temperatures below 80^{o}C (Picot and Lacroix, 2004).

1.7.3 Freeze drying of probiotics

Freeze-drying involves the removal of water from frozen cell suspension by sublimation under reduced pressure (Malik, 1990). Sublimation is the process whereby water is removed as water vapour directly from ice, without passing through the liquid state (Klamathbluegreen, 2003). Freeze drying is well suited for preservation of sensitive biological material because freezing slows or stops most chemical reactions. The process occurs under vacuum and in the absence of oxygen which make it impossible for oxidative reactions to occur. It is regarded as the gold standard of drying methods where the preservation of biological activity, flavour, aroma and/or chemistry is important (Klamathbluegreen, 2003).

Freeze-drying is a convenient method for the preservation and long term storage of a wide variety of microorganisms. Special precautions are needed for the preservation of microorganisms sensitive to desiccation, light, oxygen, osmotic pressure, surface tension and other factors. Effective protective agents, for example skim milk and meso inositol, honey or glutamate or raffinose are used to suspend cells to be freeze dried in order to

protect them against freezing and drying injuries. Along with the protective agents mentioned, anaerobic bacteria which are sensitive to aerobic freeze drying can be preserved using activated charcoal (5 % w/v) in the suspending media (Malik, 1990).

1.8 Supercritical fluids

A supercritical fluid is a substance that, at temperatures and pressures greater than its critical temperature and pressure, is a gas-like, compressible fluid that takes the shape of its container and fills it. The critical temperature is the temperature at the critical point and is the temperature above which a substance cannot exist as a liquid at any pressure. The critical pressure is the pressure at the critical point and is the pressure that will cause liquefaction of a gas at the critical temperature (Demirbaş, 2001). The supercritical state is when the temperature and pressure of a substance are raised over these critical values. In this state, the distinction between the liquid and gas phases disappears and the fluid can no longer be liquefied by raising the pressure nor gas be formed on increasing the temperature (Sihvonen et al., 1999).

A supercritical fluid has liquid like densities (0.1 – 1 g/ml) and solvating power, although it is not a liquid (Demirbaş, 2001). Physicochemical properties such as density, diffusivity, dielectric constant and viscosity can be easily controlled by changing pressure and temperature without crossing phase boundaries (Sihvonen et al., 1999). Supercritical fluids are compressible, and small pressure changes produce significant changes in their density and in their ability to dissolve compounds (Demirbaş, 2001). A supercritical fluid has a higher diffusion coefficient, lower viscosity and surface tension than a liquid solvent, and this translates into more favourable mass transfer (Sihvonen et al., 1999; Demirbaş, 2001).

The expense of organic solvents, environmental factors and the requirements of extra pure products by the medical and food industries have increased the need to develop new processing techniques (Sihvonen et al., 1999). The most widely used compound in pharmaceutical, nutraceutical and food applications is carbon dioxide though there are a

number of compounds e.g. ethane, propane, acetone, etc. (Demirbaş, 2001) that can be used as fluids in supercritical techniques. Carbon dioxide is a good solvent as it is environmentally benign (Hénon et al., 1999), non-toxic, non-flammable, inexpensive (Hénon et al., 1999; Sihvonen et al., 1999; Demirbaş, 2001), non corrosive (Demirbaş, 2001), easy to remove from product (Sihvonen et al., 1999) and easily recyclable (Hénon et al., 1999). Its use is also justified by its wide availability and relatively low critical temperature and pressure (31 ^{o}C and 72±1 bar) (Hénon et al., 1999; Sihvonen et al., 1999; Demirbaş, 2001) and heat of vaporization at 294 K is only 0.1512 MJ/kg (Demirbaş, 2001). As the pressure and temperature is varied, CO_2 assumes a density and polarity range similar to a solvent strength of pentane to benzene (Demirbaş, 2001). Supercritical CO_2 is a non-polar solvent and can therefore not be used for dissolving polar molecules (Sihvonen et al., 1999). Though the non-polar nature of CO_2 limits its solubility of polar reactants, its combination of liquid like density and gas like viscosity and diffusivity leads to high reaction rate and easy recovery of products (Sarrade et al., 2003). Although CO_2 has no dipole moment, it does have a quadropole moment, which allows for specific interactions with some molecular groups such as carboxyl, ether and ester groups, leading to increased compatibility of molecules containing such groups with supercritical CO_2. Supercritical fluids have been widely used in extraction and recovery of high value compounds. Experience accumulated in recent years on the use of supercritical fluids and their processes have indicated that it is possible to explore and envision their uses beyond the common practice of extraction (Sarrade et al., 2003). Supercritical fluid technologies can also be applied in making new innovative products. One of the very promising areas of research is microencapsulation of drug molecules, which are used for controlled drug release in the human body (Sihvonen et al., 1999).

Development of an encapsulation technology that overcomes the problems encountered using the current technologies would enable the protection and preservation of sensitive substances, improved viability, effectiveness and shelf life. The main objective of this research is therefore to investigate the suitability of the novel method of encapsulation based on the formation of an interpolymer complex in supercritical carbon dioxide, for the encapsulation of probiotics for food and pharmaceuticals applications.

1.9 Methods for detection of probiotic cultures

The methods used for detection of viable probiotic cells include conventional plate counts (culture dependent) and molecular techniques (culture-independent). Though traditional plate counting techniques are generally criticized due to the possibility of underestimation of numbers as a result of clumping of cells (Lahtinen et al., 2006) and unsuitability (inappropriateness) of media for growing of viable but non-culturable cells (Lahtinen et al., 2006; Veal et al., 2000), there is no method that can replace this yet though a number of methods are being tried. New methods include molecular based techniques such as quantitative real-time polymerase chain reaction (PCR), fluorescent in situ hybridization (FISH) and LIVE/DEAD *BacLight* bacterial viability kits (Veal et al., 2000; Boulos et al., 1999). All these methods have their own disadvantages. For example, L/D kits and real time PCR are based on bacterial DNA which is not only present in live cells but can also be retained by dead cells in significant amounts. Both PCR and FISH are not independent as they require determination of a standard curve which is determined most of the times using standard plate counts. PCR requires expensive reagents which cannot be afforded by everyone in the industry. Detection limits for PCR and FISH are relatively high, being about 10^4 cells/ml and 10^6 cells/ ml, respectively. FISH is based on detection of 16s rRNA whose presence is not a direct proof of metabolic activity but rather an indication of potential viability (Biggerstaff, 2006). A recent study by Lahtinen et al., 2006 indicated the limitation of real-time PCR and FISH with regards to viability whereby counts of bacteria decreased but PCR and FISH results remained higher over the experimental period. The authors indicated that results showed that degradation of DNA had not occurred and rRNA levels remained high enough for the cells to still be detected. The intensity of rRNA in dead cells may still be strong enough for visually counting (detection) though it is expected to decrease upon cell death. Thus, the RNA content of the cell detected by fluorescent probes cannot be regarded as reliable indicator of cellular viability (Vives-Rego et al., 2000). Therefore, for the purposes of this study, detection of viable cells was mainly done using conventional plate counts on selective media.

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CHAPTER 2

Viability of probiotic cultures from yoghurt samples randomly selected from South African retail stores

2.1 ABSTRACT

Incorporation of probiotics into dairy fermented products is now a well known practice. It has developed due to an increase in the number of studies reporting human health benefits coupled with regular intake of these vital microorganisms. However, several studies using both culture-dependent and -independent methods for detection and identification of the probiotic microbes, reported not only a problem with survival and stability of these microbes, but also in some cases their absence in products that claim to contain them. The aims of this study were to determine viability of cultures in commercial South African probiotic yoghurts and to determine if product labels specified the probiotic cultures and their levels in colony forming units (cfu) by the end of the shelf life. Eleven yoghurt samples randomly collected from retail stores were serially diluted in sterile ¼ strength Ringer's solution after thawing at room temperature. Appropriate dilutions were pour-plated in triplicate on de Man, Rogosa and Sharpe (MRS) + maltose agar and MRS agar supplemented with 0.05 % cysteine hydrochloride for lactobacilli and bifidobacteria, respectively. All plates were incubated at 37 ^{o}C for 72 h in anaerobic jars with Anaerocult C for lactobacilli and Anaerocult A for bifidobacteria. All samples showed presence of high numbers of *Lactobacillus* while only six (54.5 %) of the samples showed presence of bifidobacteria. None of the samples adequately identified probiotic organisms on their labels to species level, while only two (18 %) specified the numbers of viable bacteria as cfu/mℓ. Generally, there was a problem of stability of probiotic bifidobacteria cultures in products and therefore a need for development of a method that will improve survival and hence shelf life of probiotic products.

Keywords: Probiotics, Bifidobacteria, *Lactobacillus*, yoghurt,

2.2 INTRODUCTION

The normal intestinal microflora is the first line of defence against infections. The microflora assist in absorption of nutrients from food, and it is therefore vital for good health (Holzapfel and Schillinger, 2002). The normal microflora is not permanently stable. It can be changed by a number of factors that reduce numbers of viable microbes and thus rendering it deficient as an infection barrier. These factors include among others starvation, poor diet, use of antibiotics, stress, travelling and ageing (Havenaar and Huisint'Veld, 1992; Mitsuoka, 1996; Fooks et al., 1999).

It was observed by Mechnikoff in 1907 that Bulgarian peasants were healthy and lived longer due to their consumption of yoghurt. This led the scientific world to look at the link between the intestinal microflora and the prevention of human diseases (Oliveira et al., 2001; Schrezenmeir and de Vrese, 2001; Teitelbaum and Walker, 2002). The intentional introduction of strains of beneficial bacteria in order to starve off pathogenic microbes has since been adopted (Mombelli and Gismodo, 2002). Various *Lactobacillus* spp. and *Bifidobacterium* spp. collectively known as AB cultures are incorporated into commercial probiotic products such as yoghurts and other fermented foods (Theunissen et al., 2005. These probiotic bacteria are becoming more and more popular as their contribution to good health is reported. These reports also increased people's knowledge on benefits of probiotics, leading to acceptance of these cultures (Desmond et al., 2002).

Probiotic microorganisms have been shown to help in the treatment of several diseases such as diarrhoea (Fooks et al., 1999 ; Marteau et al., 2001 ; Teitelbaum and Walker, 2002), cancer (Fooks et al., 1999; Macfarlane and Cummings, 1999; Marteau et al., 2001; Miguel, 2001; Schrezenmeir and de Vrese, 2001), coronary heart disease (Fooks et al., 1999; Schrezenmeir and de Vrese, 2001), modulating immune responses (Holzapfel et al., 1998; Macfarlane and Cummings, 1999) and alleviating the symptoms of lactose intolerance (Fooks et al., 1999; Miguel, 2001; Elliott and Teversham 2004).

It is very important that probiotic cultures retain their viability in sufficient numbers for them to confer health benefits on the host (Salminen et al., 1996; Holzapfel et al., 1998; Macfarlane and Cummings, 1999; Miguel, 2001; Teitelbaum and Walker, 2002). Thus, actions of beneficial bacteria, and not just their presence in fermented milk products, are responsible for positive effects (Schrezenmier and de Vrese, 2001; Teitelbaum and Walker, 2002). Dairy food products incorporating bifidobacteria are now well established in the market (Doleyres and Lacroix, 2005). However, it has been documented that not all fermented milk products available in retail stores, actually contain viable beneficial bacteria. It has been found that labels do not clearly state the contents and probiotic effects of the product, nor do the labels state the actual number of viable microorganisms that can be expected in the product. Labels generally have an exceptionally poor correlation with the actual content (Elliott and Teversham, 2004; Huff, 2004).

In the year 2004, the South African probiotic industry was reported as being worth R45 million per annum. This was equal to over a million doses taken per year with 30 000 doses of probiotics taken daily. With probiotics being such a significant industry, it is very essential that these products deliver what they should to the consumers. The main objectives of this study were therefore to:

- Check clarity of product labels with regard to proper scientific names (genus, species) of incorporated probiotic microorganisms, and levels (cfu/g or cfu/ml) at the end of shelf life, as required
- Enumerate viable *Lactobacillus* and *Bifidobacteria* present in probiotic yoghurts using conventional plating techniques
- Compare levels of viable cultures obtained in the recent study with those claimed on product labels.

2.3 MATERIALS AND METHODS

2.3.1 Sample collection and storage

Yoghurt samples were collected at random from various retail stores. Frozen and drinking yoghurts of various flavours from different manufacturers were collected. The samples were stored at 4 °C before analysis. All analyses were carried out within 24 h of sampling. The samples were left at room temperature for 30 min before analysis.

2.3.2 Bacterial enumeration

1 mℓ of each yoghurt sample was suspended in 9 mℓ of sterile ¼ strength Ringer's solution. Ten fold serial dilutions of the resulting suspension were prepared in sterile ¼ strength Ringer's solution to obtain a suitable dilution. For viable *Bifidobacteria* counts, 0.1 mℓ of the appropriate dilutions was pour plated on MRS-agar supplemented with 0.05 % cysteine-hydrochloride. For viable *Lactobacillus* counts, 0.1 mℓ of the appropriate dilutions was pour plated on MRS-agar. All the dilutions were plated out in triplicate. Plates were incubated in anaerobic jars with Anaerocult A gas-packs at 37 °C for 72 h. Anaerobic conditions were verified using an anaerobic indicator, Anaerotest strips (Merck). After incubation, the visible colonies were counted and expressed as colony-forming units per milliliter (cfu/mℓ), representing the number of viable *Bifidobacteria* and *Lactobacillus* present in each yoghurt sample.

2.4 RESULTS AND DISCUSSION

It is stipulated in the SA health and food draft regulations that labels of probiotic products should indicate viable counts of bacteria per gram of product at the end of shelf life and also give full scientific name of the probiotic species present in the product (Anonymous, 2002). Earlier reports have already highlighted the importance of documentation of viability, colony counts and species identification of organisms in probiotic products

(Marcon, 1997). These reports also indicated the inaccuracy of labelling of some probiotic products. Results of the current study on labels of probiotic products support this finding.

Table 2.1: Information from South African probiotic yoghurts and counts obtained using conventional plate counts

Product	Microorganisms claimed on the label	Counts claimed on the label	*Bifidobacteria* counted	*Lactobacillus* counted
		cfu/mℓ		
1	"Live and active NitriPlus cultures"	NS	NG	1.51×10^9
2	"Yoghurt cultures"	NS	3.80×10^8	1.16×10^9
3	"HOWARU Bifido probiotic cultures"	1×10^6	8.10×10^7	5.33×10^9
4	"HOWARU Bifido probiotic cultures"	1×10^6	NG	5.00×10^9
5	"Contains live AB cultures"	NS	1.89×10^{10}	2.39×10^9
6	"AB cultures"	NS	2.09×10^8	1.02×10^{10}
7	"Live AB cultures"	NS	NG	1.52×10^{10}
8	"Abkhasian unique live cultures"	NS	1.41×10^7	2.18×10^9
9	"Live AB cultures/ AB yoghurt cultures"	NS	1.21×10^8	9.46×10^8
10	"Live AB cultures"	NS	NG	1.93×10^9
11	"Live Probiotic AB cultures"	NS	NG	5.40×10^9

cfu= colony forming units, NS = Not specified, NG = No growth

Information from the labels of products and the results of the counts obtained in this study are summarized in Table 2.1. This study found that information given on labels of all eleven yoghurt samples collected was insufficient. None of the samples packaging gave proper species identification for the probiotic incorporated i.e. none of the products gave full names (identification) genus and species, of the incorporated cultures, while nine of the samples did not specify microbial counts. Only two of the analyzed products

(3 and 4) (Table 2.1) indicated numbers of organisms present. The label indicated that the products contained about 1 x 10^6 cfu/mℓ. Both products were from the same manufacturer. Even though the manufacturer in this case tried to comply with the requirements, the information given was still not complete. Firstly, it was not clear whether the number given was referring to the counts of all the cultures present in the product, or whether each of the organisms was present at the levels indicated. Secondly, it was not explicit whether the levels stated on the product label were those present or added during manufacturing or whether it was the numbers of bacteria that will be available at the end of shelf life. This leaves out very important information that should be given to consumers as it is desired that products contain sufficient numbers of viable probiotic bacteria to confer health benefits at the time of use and not only at the time of manufacture (Reid, 2003). Results of the current study correlated with findings by Theunissen et al. (2005) who found that there was a general inaccuracy in identification and naming of probiotics presented on SA probiotic products.

All the analyzed samples contained high numbers of viable *Lactobacillus*. The levels of lactobacilli in products ranged between 9.46 x 10^8 cfu/ml to 1.52 x 10^{10} cfu/ml (Table 2.1). Bifidobacteria were detected from only six of the products tested. The counts of bifidobacteria were however lower than those of lactobacilli in all the samples. Results indicated that survival of lactobacilli was generally better than that of bifidobacteria. Bifidobacteria are reported to require an anaerobic environment and a neutral pH to survive and maintain levels greater than 10^6 cfu/ml that is adjudged as the requirement to provide therapeutic benefits (Boylston et al., 2004). Schillinger (1999) also found that lactobacilli remained at high levels until their "best before use" period indicating relative stability of cultures. The results of this study correlated with observations from other studies (Micanel et al., 1997, Shah et al., 2000, Vinderola et al., 2000, Huff, 2004, Elliot and Teversham, 2004).

Elliot and Teversham (2004) analysed nine South African probiotic products using both culture dependent and independent methods. Their study found that four of the products did not show presence of either lactobacilli or bifidobacteria. In the recent study it was

found that all products that contained both lactobacilli (9.46 x 10^8 - 1.52 x 10^{10} cfu/ml) and bifidobacteria (1.41 x 10^7 - 3.80 x 10^8 cfu/mℓ), contained them in numbers high enough to confer beneficial effects in the host (Table 2.1). The numbers of viable bifidobacteria and lactobacilli from all the products containing them were greater than the suggested minimum as required by SA legislation and international standards. However, in the study by Elliot and Teversham (2004) it was found that only three of the five products that contained viable cultures had sufficient bacteria for probiotic effect. On the other hand, Theunissen and Witthuhn (2004) also analyzed 20 different South African probiotic products for their probiotic content. There researchers found that 55 % of the products contained all cultures claimed on their labels but no bifidobacteria could be detected from 45 % of the products. All these studies showed poor correlation between labels and contents of SA probiotic products, and problem of culture viability in the food products, especially bifidobacteria. The problem of viability is however not encountered in SA products only but has been found by researchers in probiotic products from elsewhere in the world.

Micanel et al. (1997) investigated the viability of cultures in four Australian commercial probiotic yoghurts over the shelf life of the products, with shelf life expectancy (use-by date) in each instance varying between 5 and 6 weeks. The numbers of *Lactobacillus acidophilus* in the products varied widely. One product retained levels of >10^7, another >10^6 cfu/g. The third product reduced slowly, but maintained levels of >10^5 cfu/g while no viable organisms (<10^3 cfu/g) were detected in the fourth product. Of the three products that claimed to contain bifidobacteria, one maintained high levels of >10^6 cfu/g, another showed a steep decline in numbers from 1.5 x 10^5 to <10^3 cfu/g within 2 weeks of manufacture while no viable cultures were detected in the third.

A randomised double blind study by Huff (2004) on North American products purchased from six different retail stores in the lower British Columbia mainland, to assess whether commercially prepared probiotic products claiming to contain *Lactobacillus*, contained viable organisms as claimed by their manufacturers, found similar problems as observed in the recent study. None of the products matched their labelled microbiological

specifications qualitatively and quantitatively. Of the ten products tested, two did not show any growth, four did not grow the *Lactobacillus* species listed on their labels while the remaining four contained species not listed on labels. It was found that commercially available over the counter products were inaccurately labelled and that some of the products tested contained dead bacteria only.

The results indicated that consumers all over the world are buying products that are not what their manufacturers claim them to be. Death of probiotic bacteria in products is due to a number of factors including H_2O_2 produced by starter bacteria, oxygen content, pH, storage environment and concentration of metabolites such as lactic and acetic acids (Lourens-Hattingh and Viljoen, 2002; Talwalkar and Kailasapathy, 2003; Akalin et al., 2004). When H_2O_2 is present in cells it inhibits metabolism of sugar by bifidobacteria, through blockage of the key enzyme, fructose-6-phosphofructokinase (Talwalkar and Kailasapathy, 2003). Post acidification or over-acidification occurs during refrigerated storage resulting in a decrease in pH (Lourens-Hattingh and Viljoen, 2002). This often reduces the pH of yoghurt during storage to levels as low as 3.6. This is said to be the most important factor in bifidobacteria mortality, with storage temperature having a secondary effect (Kailaspathy and Rybka, 1997). It was found that uncontrollable growth of strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Kailaspathy and Rybka, 1997; Lourens-Hattingh and Viljoen, 2002) at refrigerated temperatures leads to accumulation of D-lactic acid in the product. Bifidobacteria are susceptible to acids and oxygen, consequently, their counts in yoghurts supplemented with these organisms may easily decrease during storage (Kailaspathy and Rybka, 1997). The increase in titratable acidity or decrease in pH during the storage of yoghurt could be attributed to residual fermentation (Dave and Shah, 1997). As bifidobacteria are strict anaerobes, oxygen adversely affects their growth or viability (Kailaspathy and Rybka, 1997; Talwalkar and Kailaspathy, 2003). They are more vulnerable to the deleterious effects of oxygen than *L. acidophilus*. Presence of oxygen results in accumulation of oxygenic metabolites such as superoxide anion (O_2^-), hydroxyl radicals ($OH^\bullet$) and H_2O_2. The oxygenic metabolites are toxic to bacterial cells and their toxic effects damage these cells and finally result in their death (Talwalkar and Kailasapathy, 2004; Talwalkar et al., 2004). Bifidobacteria

can also undergo physiological changes due to exposure to oxygen, whereby their cells become longer than usual, with rough surfaces and require extended incubation before they adapt and start growing in culture media (Talwalkar and Kailasapathy, 2004). Oxygen can be introduced into yoghurts at various steps during and after production. During the production process, the mixing and agitation that occurs at steps such as homogenization, cooling and starter culture inoculation can incorporate atmospheric oxygen into the product. After production, atmospheric oxygen can enter into the product during filling and packaging. Oxygen also diffuses into the product through the packaging material during storage (Talwalkar and Kailasapathy, 2004; Talwalkar et al., 2004).

2.5 CONCLUSIONS

Most of the South African probiotic yoghurts available in retail stores were not properly labeled i.e. did not specify probiotic levels in (cfu/g or cfu/ml) or bacterial names with genus and species, of cultures present. There was a poor correlation between the actual contents of most of the analysed South African probiotic products available in retail stores and their label claims. Lactobacilli generally survived better in probiotic yoghurts than bifidobacteria. The problem of survival of bifidobacteria in products needs serious attention. To ensure that needs of consumers are met, research into methods that would increase the survival of probiotic cultures in products, especially bifidobacteria, must be undertaken.

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CHAPTER 3

Investigation of the efficiency of encapsulation of probiotics in an interpolymer complex in supercritical carbon dioxide using scanning electron microscopy

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3.1 ABSTRACT

Probiotics, beneficial microorganisms, must be available in certain numbers for them to produce their beneficial effects. The problem of low viability and stability of probiotics is well known worldwide. Microencapsulation, a technique for coating or protecting sensitive actives from detrimental environmental factors, has been used by various researchers in an attempt to solve this problem. However the methods used for microencapsulation still generally involve exposure of probiotics to water or other solvents, heat, oxygen, etc. during the encapsulation process, which compromises the stability of probiotic cultures. A novel method of encapsulation using formation of an interpolymer complex in supercritical carbon dioxide was developed. This study reports on the use of scanning electron microscopy (SEM) to investigate the efficiency of the newly developed encapsulation method in terms of the effect of the encapsulation process on the cell's morphology. The effect of the encapsulation process on stability of the bacterial cells was also investigated. SEM images indicated liquefaction of both polymers (poly (vinyl pyrrolidone) (PVP) and poly (vinyl acetate-*co*-crotonic acid)) (VA-CA) in $scCO_2$. Complete encapsulation of *Bifidobacterium lactis* cells was achieved, indicated by absence of bacterial surfaces on the encapsulated particles. Encapsulation of *B. lactis* within the interpolymer complex produced smooth textured particles that were less porous when compared to non-encapsulated freeze-dried bacteria powder. Pores may allow contact between cells and unfavourable environmental factors. No visual morphological changes to *B. lactis* cells were observed due to the encapsulation process. Survival of non-encapsulated cells and cells that were exposed to the encapsulation process was similar. Thus, the encapsulation process did not negatively affect stability and viability of bacterial cells. The successful encapsulation of the bacterial cells within the interpolymer complex, the absence of changes to cell morphology and the use of FDA-approved polymers give the technology potential for application in the food and pharmaceutical industries.

Keywords: interpolymer complex; supercritical carbon dioxide; encapsulation; probiotics, *Bifidobacterium lactis*, poly (vinylpyrrolidone), poly (vinyl acetate co-crotonic acid)

3.2 INTRODUCTION

The World Health Organization and Food and Agriculture Organization of the United Nations (FAO/WHO, 2001) define probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Leahy et al., 2005). High numbers of viable probiotic cultures are necessary for production of beneficial health effects (Salminen et al., 1996; Holzapfel et al., 1998; MacFarlane and Cummings, 1999; Miguel, 2001; Teitelbaum and Walker, 2002; Talwalkar et al., 2004). Maintanance of viability of the cultures during processing and storage presents a serious technological and marketing challenge for incorporation of these cultures in functional foods by industries.

It is difficult and sometimes even impossible for manufacturers to back up claims on their product labels due to unstable shelf lives of probiotic cultures (Siuta-Cruse and Goulet, 2001). Several market surveys reported a decline in the counts of *Lactobacillus acidophilus* and *Bifidobacterium spp*. during the shelf life of commercial products containing probiotics, with cell numbers significantly lower than the recommended levels at the end of shelf life (Micanel et al., 1997; Vinderola et al., 2000; Elliot and Teversham, 2004; Huff, 2004). The survey done in this project on fermented probiotic products available on South African retail store shelves indicated that there was a problem of survival of probiotics, especially bifidobacteria, in products.

Over the years, microencapsulation, the process whereby the core material is captured in a shell or coating for controlled release, has been used. Through microencapsulation, cells can survive processes such as freezing and freeze-drying better, as well as be protected from attack by bacteriophages (Krasaekoopt et al., 2003). Protection of probiotics by encapsulation in hydrocolloid beads has been investigated for improving their ability in food products and the intestinal tract. Researchers favour the use of extrusion and emulsion for encapsulating microbial cells (Krasaekoopt et al., 2003). The disadvantage of using emulsions is that production of large quantities of beads and

washing them free of oil is difficult (Stormo and Crawford, 1992). It is also difficult to produce gel beads at a large scale due to a number of reasons as discussed in Section 1.7.2.2 (Krasaekoopt et al., 2003; Picot and Lacroix, 2004).

Several techniques such as spray drying and fluidized bed drying are used for encapsulating the cultures and converting them into a concentrated form. One of the disadvantages of these techniques is that the bacteria are completely released in the product. Thus the cells are not protected from the product environment and during passage through the stomach or intestinal tract (Krasaekoopt et al., 2003). Toxic organic solvents accumulate in microbial cells and kill them through destruction of the functional properties needed for their survival (Sardessai and Bhosle, 2002; Matsumoto et al., 2004).

Organic solvents are not only toxic to cells, they are expensive as well. Negative effects of environmental factors such as moisture, temperature and oxygen on probiotic cultures must be minimized. Medical and food industries require ultra pure products. All these reasons indicate that new peocessing techniques that can fulfil all these requirements must be developed (Vasishtha, 2003). An encapsulation technology that would overcome the problems posed by current technologies, enabling protection and preservation of sensitive substances, improvement of their viability, effectiveness and shelf lives should therefore be developed.

The challenges in developing commercially viable encapsulated products depend on:

- Selection of appropriate shell formulation from FDA-approved GRAS (generally recognized as safe) materials
- Selection of the most appropriate process to provide the desired morphology
- Stability and release mechanism
- Economic feasibility of large scale production, including capital, operating and other miscellaneous expenses, such as the transportation cost, regulatory cost and downtime losses.

Supercritical fluids (SCFs) are fluids heated to temperatures and pressures above their critical temperature and pressure. They are able to solubilize compounds and can penetrate low porosity materials (Demirbas, 2001). SCFs have gas-like diffusivity and liquid-like densities (Reverchon and Porta, 2001). Though they were originally used for extraction, experience accumulated in recent years on their use and processes indicated the possibility to explore and envision their use beyond the common practice of extraction (Reverchon and Porta, 2001; Sarrade et al., 2003). Supercritical fluid technologies can also be applied in making new innovative products (Sihvonen et al., 1999; Reverchon and Porta, 2001). Encapsulation of drugs for release at specific sites in the human body is one of the new areas for application of supercritical fluid technology (Sihvonen et al., 1999).

Supercritical carbon dioxide ($scCO_2$), has received increasing attention due to its cost effectiveness and environmental friendliness (Bae et al., 2004; Novik et al., 2006). The relatively low critical parameters (T_c = 31.1 ºC and P_c = 73.8 bar[1]) of $scCO_2$ lends it towards processing of pharmaceuticals and other materials that are sensitive to temperature, solvents, oxygen, water, etc. such as proteins, labile drugs and bacteria (Reverchon and Porta, 2001; Bae et al., 2004). An additional advantage of using SCFs as solvents, particularly in pharmaceutical applications is that there is no residual solvent in the final product (Corrigan and Crean, 2002). Typical applications of $scCO_2$ in biotechnology include micronization of drugs and encapsulation of sensitive actives for controlled release of the immobilized material (Jung and Perrut, 2000; Fages et al., 2004; Ginty et al., 2005; Yeo and Kiran, 2005).

Most polymers are not sufficiently soluble in or compatible with $scCO_2$ and can thus not be processed using it as a solvent or plasticiser. Different approaches can be used to overcome the problem of insolubility and incompatibility between polymers and $scCO_2$, though they are typically not allowed in food and pharmaceuticals. The different approaches used and reasons for their limitation in these industries are given in Table 3.1.

Polymers with complementary sites/ molecular groups can interact with each other in solution to form physical networks by interpolymer complexation (Tsuchida, 1994; Henke et al., 2005). Interpolymer complex assemblies form through any of four fundamental attractive interactions, namely electrostatic attraction, hydrogen bonding, hydrophobic interaction and Van der Waals forces (Henke et al., 2005). It has been shown that hydrogen bonding (Tilly et al., 1994) and dipole-dipole interactions (Ekart et al., 1993) can occur in $scCO_2$. $scCO_2$ has largely been used in the food industry for extraction of labile food components and in pharmaceutical industries for extraction and purification of vitamins. Recently Novik et al. (2006) reported the use of $scCO_2$ in the probiotics field for extraction of glycolipids from *Bifidobacterium adolescentis* 94 BIM. However, the formation of interpolymer complexes in $scCO_2$ and its application in encapsulation of probiotic bacterial cells was to our knowledge, reported for the first time by Moolman et al. (2005).

The main objectives of this study were therefore to investigate the efficacy of this novel encapsulation technique based on interpolymer complexation in $scCO_2$ using SEM, the effect of encapsulation on cell morphology and to determine the effect of the encapsulation process on stability of bacteria using conventional plating techniques.

Table 3.1: Approaches to overcoming incompatibility between $scCO_2$ and most polymers (from Moolman et al, 2006)

Approach	Elaboration	Limitations	Reference
Changing polymer design	Incorporation of "CO_2-philic" functional groups in new polymers	FDA approval needed for new polymers	Sarbu et al., 2000
Surfactants	Addition of CO_2 soluble surfactants	FDA approval needed for surfactants	Hoefling et al., 1993; McClain et al., 1996; Yazdi et al., 1996
Cosolvents	Addition of cosolvents , e.g. methanol or ethanol, to increase the solvation power of $scCO_2$	Reintroduces requirement for use of a solvent-many actives are sensitive to solvents	Ekart et al., 1993; Kazarian et al., 1998; Mishima et al., 2001; Corrigan and Crean, 2002
Mixtures of SCFs	The use of 2^{nd} SCF to enhance polymer processability	No obvious 2^{nd} SCF with desired combination of properties (low/no toxicity, low critical temperature and pressure, low cost, etc.)	
Gas anti-solvent technique	Use of $scCO_2$ 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 of low molar mass and low polarity polymers	Polymers are more amenable to $scCO_2$ processing	These polymers generally have low mechanical integrity and/or barrier properties	Rindfleisch et al., 1996
Use of fats/waxes for encapsulation	Fats, waxes and oils are generally soluble in $scCO_2$	Limited flexibility with regards to properties	Benoit et al., 2000

3.3 MATERIALS AND METHODS

3.3.1 Bacterial cultures

Bifidobacterium lactis Bb-12 and *Bifidobacterium longum* Bb-46 were obtained as DVS sachets from CHR- Hansen.

3.3.2 Encapsulation of bacteria

Bifidobacterium cells were encapsulated using a Particles from Gas-Saturated Solution (PGSS) reactor (Fig. 3.1). All equipment was wiped with 70 % ethanol using a paper towel, and allowed to dry before contact with the materials. 2 g of PVP (Kollidon 12PF, mass-average molar mass 2 000 – 3 000 g/mol, BASF) was dried for 5 h at 80 ºC and 60 mbar (absolute) in a vacuum oven (Model VO65, Vismara) and immediately placed in a dessicator to prevent moisture absorption. A sealed packet of either *B. longum* Bb-46 (Chr. Hansen) or *B. lactis* Bb-12 (Chr. Hansen) was removed from storage at -12 ºC and allowed to warm to room temperature while sealed. 2 g of the bacteria was then ground to a powder passing through a 150 µm sieve using a coffee grinder (Model CG100, Kenwood). 6 g of VA-CA (Vinnapas C305, mass-average molar mass 45 000 g/mol, Wacker) was then added to the bacteria (together with any additives (e.g. glyceryl monostearate – Croda Chemicals, in reactions were additives were included) and the dried PVP. The blend was then ground and mixed for 1 min. The powder blend was then immediately transferred to the pre-heated 1 ℓ reaction chamber. The chamber was then sealed and flushed and pressurized with sterile filtered CO_2 (99.995% purity, Air Products) up to a pressure of 300 bar, with the temperature controlled at 40 ºC. The material was left to equilibrate for 2 h with intermittent stirring, after which the liquefied product was sprayed through a 500 µm capillary with length 50 mm, into a 10 ℓ expansion chamber that was pressure-controlled at 15 bar (gauge). Fig. 3.2 is a simplified flow diagram showing steps occurring in the encapsulation process. A clear description of the encapsulation technology is outlined in Moolman et al. (2006).

Figure 3.1: A 1ℓ PGSS reactor (Separex Equipments, France) used for encapsulation of bacteria, situated at the Polymers and Ceramics centre, CSIR, Brummeria, PTA. A= CO_2 supply, B= Pump, C= CO_2 preheater, D= Mixing chamber, E= Product chamber, F= Control panel

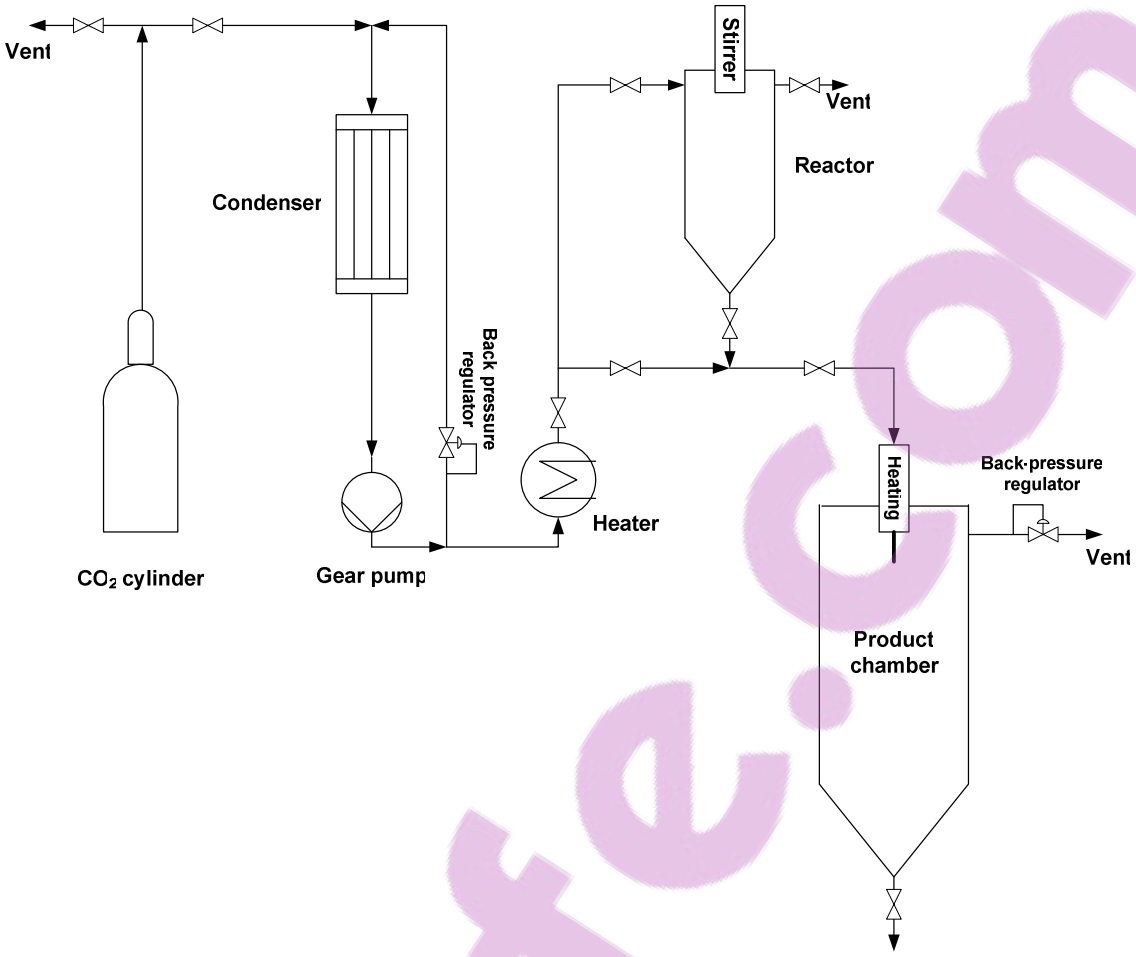

Figure 3.2: A simplified process flow diagram of the PGSS (Particles from Gas-Saturated Solution) system used to produce encapsulated probiotics

3.3.3 Scanning electron microscopy (SEM)

SEM was used to verify encapsulation of *B. lactis* cells into the polymer and release of the cells from the polymer into solution during subsequent suspension of the encapsulated material. The freeze-dried and encapsulated bifidobacteria were suspended in ¼ strength Ringer's solution. The suspended cells were filtered out using a 0.2 µm Millipore filter membrane. The cells were fixed to the 0.2 µm membrane using 2.5 % gluteraldehyde for 30 min. The fixed cells were then washed 3 x 15 min in 0.15 M-phosphate buffer. Then dehydration of the sample was done in an increasing series of ethanol as follows: 50 % (1 x 15 min), 70 % (1 x 15 min), 90 % (1 x 15 min) and 100 % (3 x 15 min). The filter

membrane was then dried in a critical point dryer for 24 h, mounted on stainless steel studs and then coated with gold plasma. The freeze-dried and encapsulated powder were put on a sticky tape on the studs and directly coated with gold plasma without undergoing any treatments for SEM. The samples coated with gold were then examined using JEOL 840 scanning electron microscope.

3.3.4 Bacterial counts

1 g of *Bifidobacteria* was suspended in 9 mℓ of sterile ¼ strength Ringers solution (pH 7). A series of dilutions up to 10^{-10} was prepared from this suspension. 0.1 mℓ of appropriate dilutions was pour plated onto De Man, Rogosa and Sharpe (MRS) agar (Merck, Pty.(Ltd)), supplemented with 0.05 % cysteine hydrochloride. Each dilution was plated out in triplicate. The plates were incubated anaerobically in anaerobic jars with Anaerocult A gaspaks (Merck Pty (Ltd.), at 37 ^{o}C for 72 h. Anaerobisis inside the jars was indicated by inclusion of Anaerocult C test strips (Merck, Pty (Ltd)). The numbers of colonies grown were counted and from these the numbers of viable cells were calculated (cfu/g). Reported values are averages of the three replicates.

3.4 RESULTS AND DISCUSSION

3.4.1 Liquefaction of polymers in $scCO_2$

SEM was used to examine whether the developed SCF encapsulation method was efficient and whether the polymers used were liquefied during exposure to $scCO_2$. SEM images of PVP and VA-CA before and after exposure to $scCO_2$ are shown (Fig. 3.3). These images indicated that liquefaction of both polymers occurred during exposure to $scCO_2$ (Fig. 3.3). Images before exposure to $scCO_2$ showed polymers as individual granules (separate loose particles with individual particles/ granule's three dimensional structure visible showing that the particles were separate) (Fig. 3.3 A, C) while those after exposure appeared as a monolithic foam (Fig. 3.3 B, D). No individual particles similar to those observed before exposure to $scCO_2$ were present. The continuous

appearance (compact layer) was the result of liquefaction of the polymers by the dissolution of $scCO_2$ in the polymers. Dissolution of $scCO_2$ in polymers is known to lower glass transition temperature (Tg) and facilitates formation of a smooth morphology (Yue et al., 2004).

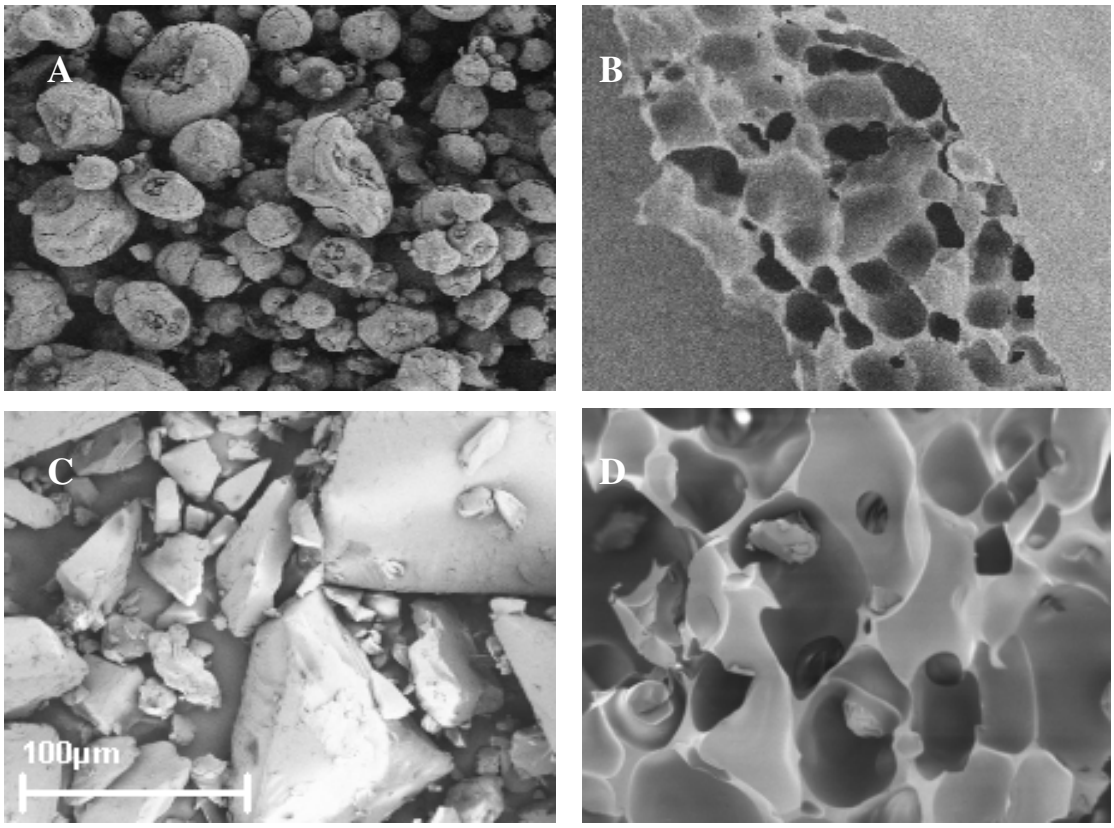

Figure 3.3: Comparison of PVP before (A) and after (B) and VA-CA before (C) and after (D) $scCO_2$ exposure

3.4.2. Interpolymer complexation and bacterial encapsulation

Encapsulation of *B. lactis* Bb-12 cells by the interpolymer complex formed between PVP and VA-CA was achieved (Fig. 3.4 B). PVP is a water soluble inert polyamide polymer with complexing properties and has been used in pharmaceutical products (Kumar et al., 1999). It has carbonyl groups while VA-CA has carboxylic acid groups (Raveendran et al., 2005). Therefore it was expected that these two groups would interact with each other through hydrogen bonding and form a complex between the two polymers. Such an

interpolymer complex was indeed formed through the formation of hydrogen bonds between the carboxylic acid groups and carbonyl groups of VA-CA and PVP, respectively (Moolman et al., 2006). Similar results were observed by other researchers (Raveendran et al., 2005). Surface characteristics or appearance of encapsulated material and freeze-dried bacterial powder were different. Even though the freeze-dried cells were clumped together, the rod-shaped individual bacterial cells forming the clumps could be seen (Fig. 3.4 A). It could be observed from the images that there was no layer around or covering the bifidobacteria cells to offer protection. Thus the non-encapsulated freeze-dried bacteria had no shield to protect them should they be exposed to detrimental factors during storage or after ingestion.

On the other hand, no bacterial cells were visible on surfaces of the encapsulated material. The encapsulated bacteria were therefore completely enclosed within an interpolymer complex formed. The ability of polymers to interact with each other in solution through secondary binding forces such as hydrogen bonds, dispersion forces and hydrophobic interactions, to form intermacromolecular complexes is well known (Henke et al., 2005). The interpolymer complex surrounding the bacteria serves as a potential barrier against detrimental environmental factors to which the probiotic bacteria are normally exposed, such as oxygen during storage and gastric acid during transit through the gastrointestinal tract.

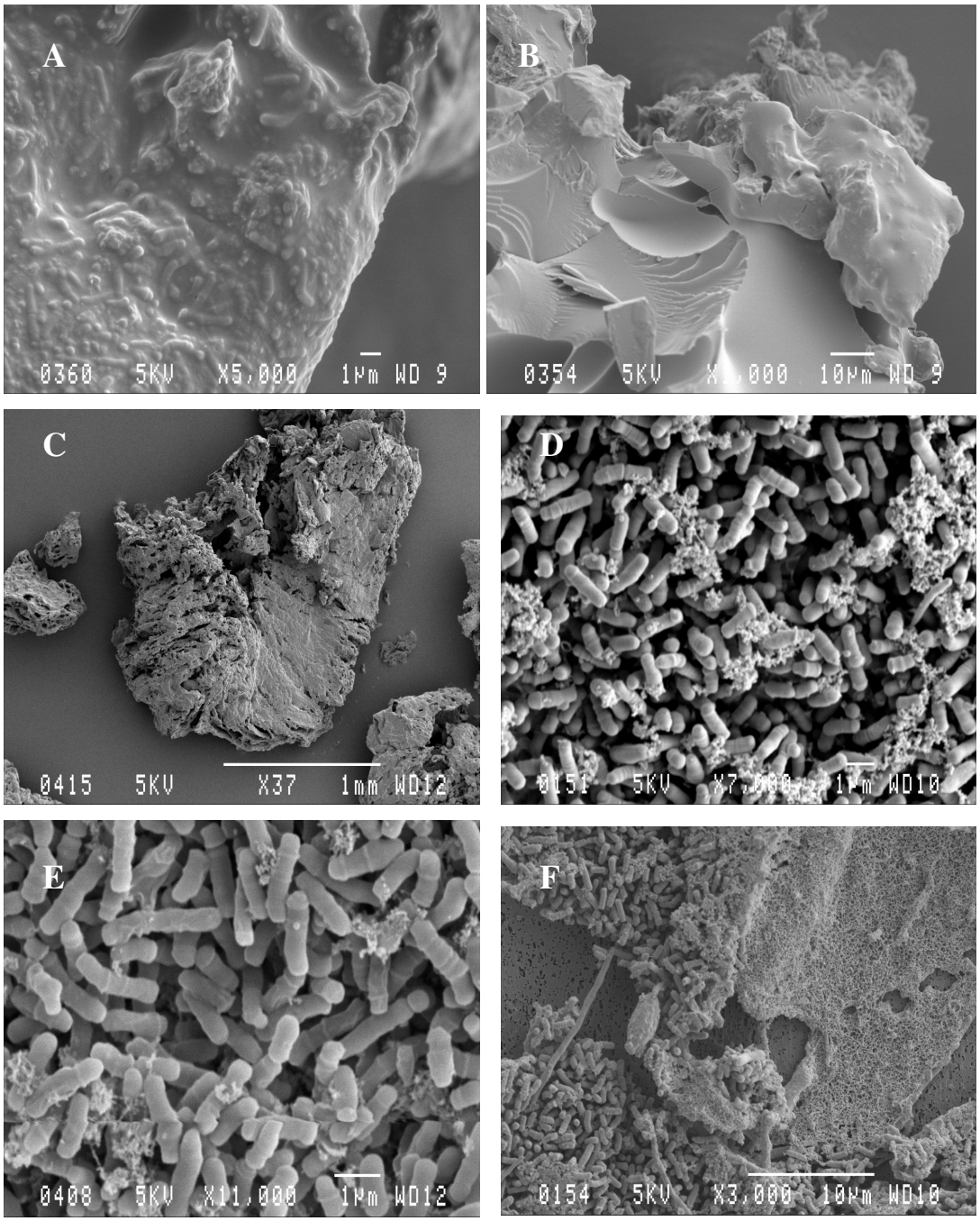

Figure 3.4: SEM images of *Bifidobacterium longum* Bb-46 cells: A and C: Non-encapsulated (powder), B: scCO$_2$ interpolymer complex encapsulated (powder), D: Non-encapsulated (suspension), E and F: scCO$_2$ interpolymer complex encapsulated (suspension)

Water insoluble dry capsules for incorporation of bifidobacteria in food products should, according to Doleyres and Lacroix (2005), have a particle size of <100 µm for stability, easier handling and storage of cultures as well as limited effects on the product texture.
The size of particles produced using the method described in this chapter can be controlled through the use of additives such as glyceryl monostearate. Addition of glyceryl monostearate reduced the particle size by more than an order of magnitude (results not shown).

3.4.3 Appearance of non-encapsulated and encapsulated cells upon suspension

Suspension of encapsulated powder in sterile distilled water (pH 6.8) released the encapsulated bifidobacteria cells from the interpolymer complex into the solution/suspension. PVP is water soluble (Kumar et al., 1999) while VA-CA is insoluble. Though VA-CA is insoluble in water, higher pH results in dissociation of the crotonic acid groups, leading to increased compatibility with water (Moolman[1], Personal communication). This then causes VA-CA to swell, causing the release of the bacteria enclosed within the interpolymer complex. The residues of the disrupted polymer were visible between the bacterial cells and on the mounting surface. When the released suspended cells were viewed under SEM no visible differences between the bifidobacteria cells released from the interpolymer complex (Fig. 3.4 E) and the freeze-dried non-encapsulated bacteria in suspension (Fig. 3.4 D) were observed. This indicated that the encapsulation process did not produce any noticeable damage or morphological changes to the bacteria. Thus it seemed that the encapsulation process did not negatively affect the enclosed cells, but this can not be concluded from the SEM results alone.
The numbers of cells captured from 1 ml of the suspension were high for both non-encapsulated and encapsulated bacteria. The method therefore allowed encapsulation of high numbers of cells which is an advantage when looking at the envisaged application of the method in probiotic foods whereby high levels of viable bacteria are required for production of beneficial effects.

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3.4.4 Viability of *Bifidobacterium longum* Bb-46 cells after $scCO_2$ processing

SEM images were able to show satisfactory encapsulation of bacteria and an unchanged physical appearance of cells upon suspension, but could not tell whether the cells were alive or dead. Plate counting technique was applied to fulfil this crucial purpose. Fig. 3.5 shows counts of bacteria over six weeks. Numbers of viable bacteria for both unprocessed and $scCO_2$ processed cells decreased over the storage time.

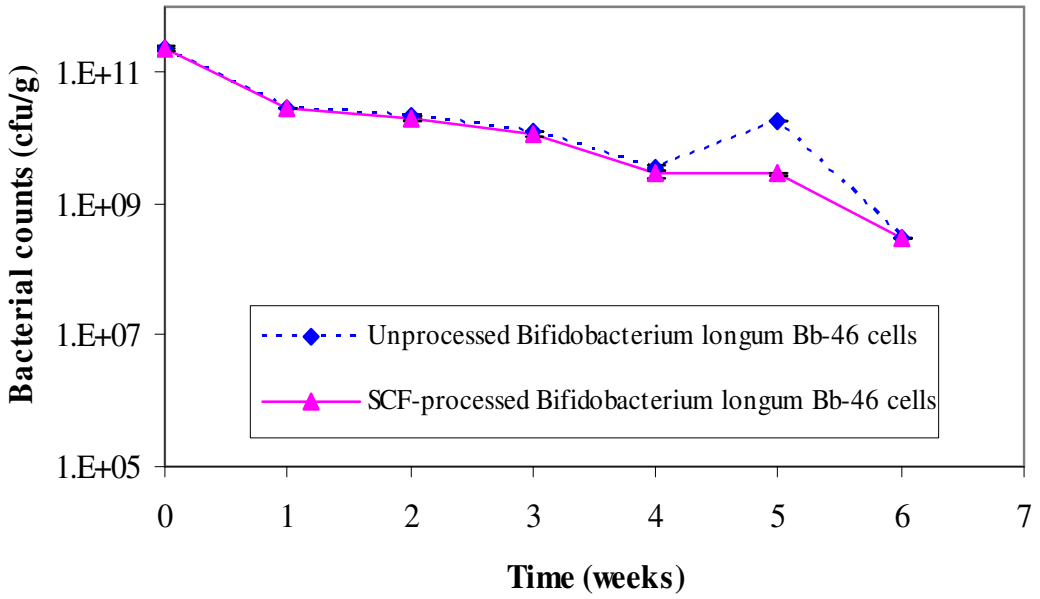

Figure 3.5: Survival of freeze-dried bacteria and freeze-dried bacteria that went through the encapsulation process during storage

The reduction in numbers of viable bacteria over time for $scCO_2$ processed and unprocessed bacteria were similar (Fig. 3.5). The counts of both $scCO_2$ processed and unprocessed cells were around 2×10^{11} cfu/g at the beginning of the trial and reduced to 3×10^{8} cfu/g after 6 weeks of storage at 30 ^{o}C (Fig. 3.5). There was no significant difference in the numbers of live bacteria for both samples over 6 weeks. The results indicated that not only did the exposure to $scCO_2$ not produce noticeable effects to the morphology of the processed bacteria, but the stability and therefore viability of the cells

was also not negatively affected. Probiotic bacteria encapsulated using the described method should thus remain viable within the interpolymer complex after encapsulation and produce beneficial effects upon release at the desired site.

3.5 CONCLUSIONS

Satisfactory liquefaction of VA-CA and PVP upon exposure to supercritical carbon dioxide was achieved. Successful encapsulation was indicated by the absence of bifidobacteria on interpolymer complex surfaces and by the release of high numbers of bifidobacteria from the interpolymer complex upon suspension. No undesirable effects such as morphological changes or reduced cell stability occurred as a result of the encapsulation process. The results therefore indicate for the first time, the potential of interpolymer complexation in $scCO_2$ for application in food and pharmaceutical industries for encapsulation of sensitive probiotic bacteria in order to protect the cells from detrimental factors leading to unwanted reduction in viability.

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CHAPTER 4

Simulated gastric and intestinal fluid survival of *Bifidobacterium longum* Bb-46 encapsulated in different interpolymer complexes formed in supercritical carbon dioxide

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4.1 ABSTRACT

Probiotics, incorporated in traditional fermented foods and as supplements, are now available to consumers as over the counter products. They are however sensitive to a number of environmental factors. Gastric acid is the main factor affecting viability of probiotics in the gastrointestinal tract. Sensitivity of probiotics, specifically bifidobacteria, to acidic environments therefore presents a challenge for their incorporation into food and pharmaceutical products. This study investigated the survival in simulated gastrointestinal fluids of encapsulated *Bifidobacterium longum* Bb-46. Both non-encapsulated and encapsulated *B. longum* Bb-46 cells were exposed to simulated gastric fluid (SGF, pH 2) for 2 h and subsequently to simulated intestinal fluid (SIF, pH 6.8) for 6 or 24 h. Samples taken after different exposure times were diluted and pour-plated using MRS-agar supplemented with 0.05 % cysteine hydrochloride. Plates were incubated anaerobically at 37^{o}C for 72 h. The interpolymer complex displayed pH-responsive release properties, with little to no release in SGF and substantial release in SIF. There was a smaller reduction in numbers of viable bacteria for encapsulated than for non-encapsulated bacteria at the end of exposure. Protection efficiency of the normal interpolymer complex was improved by addition of glyceryl monostearate (GMS) and use of gelatine capsules. An increase in GMS loading from 8 % to 60% resulted in better protection. Use of polycaprolactone (PCL) and incorporation of ethylene oxide-propylene oxide triblock copolymer (PEO-PPO-PEO) decreased the protection efficiency of the interpolymer complex, while results for beeswax were inconclusive. Interpolymer complex encapsulation has potential for protection of probiotics and therefore for application in food and pharmaceutical products.

Keywords: Probiotics, Simulated intestinal fluid, simulated gastric fluid, Interpolymer complex, *Bifidobacterium longum* Bb-46, Supercritical CO_2, Polycaprolactone, Glyceryl monostearate, ethylene oxide-propylene oxide triblock copolymer

4.2 INTRODUCTION

A wide variety of probiotic lactic acid bacteria strains are available to consumers in both traditional fermented foods and in supplement form (Kourkoutas et al., 2005). Products containing probiotic microorganisms of human origin should be able to exert a beneficial effect on the consumer's wellbeing (Schillinger, 1999). These organisms are exposed to different stresses during their production, storage and consumption, which reduce the number of viable organisms (Doleyres and Lacroix, 2005). However, probiotic strains of bifidobacteria must remain viable and metabolically active in the environment where they act, that is, they must be active in the gastrointestinal tract.

These organisms must therefore survive transit through the gastrointestinal tract and reach the colon in large quantities to facilitate colonization in the host (Kailasapathy and Rybka, 1997; Alander et al., 1999; Lian et al., 2003; Hsiao et al., 2004; Mainville et al., 2005). In the large intestine ingested bacteria compete for nutrients and adherence sites on the intestinal epithelium with already established microbiota comprising several hundreds of other bacterial species (Alander et al., 1999). The main factors affecting the viability of probiotics in the gastrointestinal tract are the acidic environment of the stomach and the presence of bile in the duodenum (Rao et al., 1989; Lo et al., 2004; Mainville et al., 2005). The sensitivity of bifidobacteria to acidic environments presents a challenge for application of these microorganisms in different industries (Hansen et al., 2002).

Several studies have shown the inability of many strains of bifidobacteria to survive acidity and bile present in the human gastrointestinal tract. Different methods such as appropriate selection of acid and bile resistant strains, two step fermentations, stress adaptation, incorporation of micronutrients and microencapsulation, for improving survival of these bacteria have been tried (Picot and Lacroix, 2004).

Protection of bifidobacteria, specifically by use of microencapsulation, has been attempted by various researchers (Rao et al., 1989; Sheu and Marshall, 1993; Cui et al., 2000; Lee and Heo., 2000; Sultana et al., 2000; Sun and Griffiths, 2000; Hansen et al., 2002; Guérin et al., 2003; Lian et al., 2003; Krasaekoopt et al., 2004; Capela et al., 2006). The different encapsulation methods aim at ensuring greater survival of probiotic bacteria under gastric conditions. Most of these methods indicated the potential for application of encapsulation of probiotic bacteria in food and pharmaceuticals as encapsulated bacteria survived better than their non-encapsulated counterparts. However, more research still needs to be done on the methods for optimum protection of encapsulated bacteria.

Additionally most technologies, although promising on a laboratory scale, present serious difficulties for large scale production (Picot and Lacroix, 2004). Most methods include a step in which the probiotic culture is present in suspension / in solution. This may negatively affect survival or compromise survival of encapsulated cells as they are sensitive to solvents and moisture. The use of solvents should be avoided in order to improve chances of survival of encapsulated probiotic cultures. None of the previous studies reported gastrointestinal survival of probiotic bacteria encapsulated in an interpolymer complex in supercritical CO_2 ($scCO_2$). Encapsulation of probiotics in an interpolymer complex in $scCO_2$ was reported for the first time by Moolman et al. (2006). The aim of this study was to investigate the survival of interpolymer complex encapsulated *Bifidobacterium longum* Bb-46 in simulated gastric and intestinal fluids, and to investigate effects of different modifications of the polymers on bacterial survival.

4.3 MATERIALS AND METHODS

4.3.1 Bacterial cultures

Bifidobacterium longum Bb-46 was obtained from DVS from CHR-Hansen. The culture was stored at -20 ^{o}C and then used as freeze-dried powder in encapsulation experiments

4.3.2 Polymer formulations

Different polymer formulations used for encapsulation of bacteria are summarized in Table 4.1.

Table. 4.1: Polymer formulations used for bacterial encapsulation.

Formulation	Different ingredients (%) w/w							Total weight (g)
	Bifidobacteria	VA-CA	PVP	PEO-PPO-PEO	PCL	GMS	Beeswax	
1	20	60	20	-	-	-	-	20
2	20	60	-	-	20	-	-	20
3	19.6	36.2	12	32.2	-	-	-	20
4	19.6	36.2	-	32.2	12	-	-	20
5	20	54	18	-	-	8	-	20
6	20	15	5	-	-	60	-	20
7	20	-	16	-	-	-	64	20
8	20	-	5	-	-	-	75	20

VA-CA = Vinyl acetate-crotonic acid copolymer (Vinnapas C305 mass-average molar mass 45 000 g/mol -Wacker Chemie)

PVP = Poly (vinylpyrrolidone) (Kollidon 12PF, mass-average molar mass 2 000 – 3 000 g/mol - BASF)

PEO-PPO-PEO = Ethylene oxide-propylene oxide triblock copolymer (Synperonic PE/F68- Uniqema)

PCL = Poly(caprolactone) (Tone P300- Union Carbide)

GMS = Glyceryl monostearate (Cithrol GMS A/S- Croda Chemicals)

Beeswax =White beeswax (White Beeswax BP- Croda Chemicals)

PVP-VA = Poly(vinylpyrrolidone-*co*-vinyl acetate) (Kollidon VA64 – BASF)

4.3.3 Encapsulation of bacteria

Encapsulation of bacteria was done using the method described by Moolman et al. (2006) with no modifications.

4.3.4 Determination of total bacteria encapsulated

1 g of encapsulated product was added to 9 ml of sterile Ringer's solution (pH 6.8) in a test tube. The tube was incubated for 6 h to allow for release of bacteria before diluting and plating out.

4.3.5 Preparation of simulated gastric and intestinal fluids

Simulated gastric juice was prepared according to Lian et al. (2003). Briefly, pepsin (Merck) was suspended (3 g/ℓ) in saline (0.5 %, w/v) and pH adjusted to 2.0 with 12 N HCl. For simulated intestinal fluid, 6.8 g monobasic potassium phosphate (Merck, SA) was dissolved in 250 mℓ distilled water and mixed. 77 mℓ of 0.2 N NaOH was added, mixed and then followed by addition of 500 mℓ of distilled water. The solution was mixed by vortexing for 30 s. Then 10 g of pancreatin was added and mixed. The pH of the resulting solution was adjusted with either 0.2 N sodium hydroxide or 0.2 N HCL to a pH 6.8. The solution was made up to 1000 mℓ with distilled water. Both solutions were sterile-filtered through a 0.45 µm filter membrane (Millipore).

4.3.6 Survival of bacteria in simulated gastric fluid

1 g of either freeze-dried or encapsulated bacteria was added to 9 mℓ simulated gastric fluid (pH 2.0) in a test tube and vortexed for 30 s for complete dispersion. Samples were taken immediately after vortexing to determine viability of bacteria. The test tubes were then incubated at 37 ^{o}C in a shaker incubator (50 rpm) for 2 h. 1 mℓ aliquots were removed from the tube at times 0.5, 1 and 2 h for enumeration of bifidobacteria. The test

tube with encapsulated material was not vortexed on sampling so as not to interfere with dispersion of or release of bacteria from the polymer matrix.

4.3.7 Survival of bacteria in simulated intestinal fluid

1 g of freeze-dried bacteria was suspended in 9mℓ of simulated intestinal fluid (37 ^{o}C) in a test tube and vortexed for 30 s for dispersion of cells. For the encapsulated cells, excess supernatant or gastric juice from the gastric survival test was discarded after taking the 2 h sample and another 1 mℓ sample for intestinal fluid test. The pellet was resuspended in 9 mℓ intestinal fluid. Initial samples for bacterial counts were taken from both tubes immediately after resuspension and vortexing, for enumeration of bifidobacteria. The tube was incubated at 37 ^{o}C in a shaker incubator at 50 rpm for 6 h. 1 mℓ samples were taken immediately after mixing, after 2, 4 and 6 h.

4.3.8 Enumeration of Bifidobacteria

Serial dilutions of the aliqouted samples taken from both survival tests were prepared in sterile Ringer's solution. 100 µl of appropriate dilutions were plated out in triplicate on MRS agar supplemented with 0.05 % cysteine hydrochloride plates. The plates were incubated at 37^{o}C for 72 h in anaerobic jars with Anaerocult A gaspaks and Anaerocult C test strips for indication of anaerobic conditions inside the jar.

4.4. RESULTS AND DISCUSSION

4.4.1 Survival in PVP:VA-CA (normal system) and PEO-PPO-PEO:PVP:VA-CA

It is well known that for probiotic microorganisms to facilitate colonization in the host they must survive the journey through the gastrointestinal tract. The organisms must be able to withstand the acidic conditions of the stomach and reach the colon in large quantities (Kailasapathy and Rybka, 1997; Alander et al., 1999; Lian et al., 2003; Hsiao et al., 2004; Mainville et al., 2005). For this reason, the encapsulated probiotic bacteria

were exposed to SGF and SIF to determine whether encapsulation could improve the survival of the bacteria under the unfavourable conditions in the gastrointestinal tract.

Bacteria were exposed to SGF for 2 h to simulate the average time for emptying half of the stomach contents reported as 90 min (Sun and Griffiths, 2000). The numbers of non-encapsulated bacteria decreased with exposure to gastric fluid (Fig. 4.1). Encapsulated bacterial numbers on the other hand increased with an increase in exposure time to simulated gastrointestinal fluids (Fig. 4.1). This was due to continous release of viable bacteria from the interpolymer matrix. Upon exposure to SGF (pH 2) the non-encapsulated bacterial levels decreased from an initial count of 6.89 x 10^{10} cfu/g to 1.62 x 10^{10} cfu/g after 2 h (Fig. 4.1). The reduction in the numbers of non-encapsulated bacteria in this study was however not as rapid/sharp as with reports on other bifidobacteria at the same pH (Hansen et al., 2002; Charteris et al., 1998). Hansen et al. (2002) reported a decrease of 3-4 log cfu/g for *B. longum* Bb-46 after 2 h of exposure to SGF, while Charteris et al. (1998) reported a decrease of 3 log cfu/ml for different bifidobacteria after 3 h of exposure to SGF (pH 2). The results were however in agreement with those of Lian et al. (2003), who found that at pH 2 -3 the decrease in the number of viable bifidobacteria was not significant.

For encapsulated bacteria, there was no growth for plates with samples obtained from the PVP:VA-CA polymer matrix throughout the 2 h of exposure to SGF (Fig. 4.1). This indicated that there were no viable bacteria released from the PVP:VA-CA interpolymer complex in the low pH environment. Thus the PVP:VA-CA matrix did not swell or disintegrate in the acidic environment, protecting the encapsulated bifidobacteria cells from the detrimental effect of the SGF. An initial count of 4.0 x 10^5 cfu/g was obtained for PEO-PPO-PEO:PVP:VA-CA (Fig. 4.1). This count increased to 1.1 x 10^8 cfu/g at the end of 2 h. (Fig. 4.1). Release of bifidobacteria from PEO-PPO-PEO:PVP:VA-CA indicated that this polymer matrix disintegrated/ swelled in the low pH of the SGF releasing some of the encapsulated cells. Thus it seemed that incorporation of PEO-PPO-PEO in the formulation affected the pH-dependant swellability of the PVP:VA-CA interpolymer complex negatively, rendering it more swellable at the low pH. Hence, the

efficiency for protection of bifidobacteria from the acidic gastric juice is reduced when these sensitive probiotic microorganisms are encapsulated with PEO-PPO-PEO as a component in the matrix.

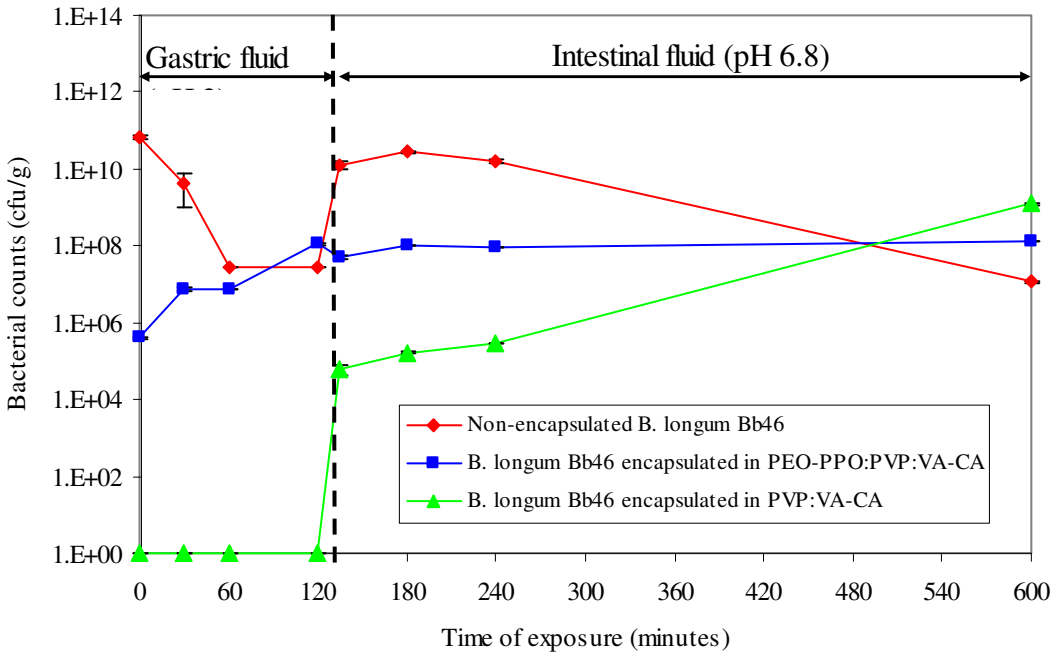


Figure 4.1: Survival of *B. longum* Bb-46 encapsulated in PVP:VA-CA and PEO-PPO-PEO:PVP:VA-CA after exposure to SGF and SIF

The numbers of viable non-encapsulated bacteria continued to decrease on subsequent exposure to SIF (pH 6.8) (Fig. 4.1). Their numbers decreased from 1.3 x 10^{10} cfu/g immediately upon exposure to 1.2 x 10^{7} cfu/g after 24 h (Fig. 4.1). However, during the first 2 h of exposure the numbers of non-encapsulated bacteria were increasing (Fig. 4.1). This increase in the number of viable cells in SIF was also observed by Picot and Lacroix (2004) who attributed it to temporary damage of bifidobacteria cells due to low pH stress. When PVP:VA-CA encapsulated material was subsequently exposed to SIF (pH 6.8), the interpolymer complex swelled as a result of the higher pH. A count of 6.1 x 10^{4} cfu/g was obtained immediately after suspension, which increased to 1.3 x 10^{9} cfu/g after 24 h (Fig. 4.1). This indicated that the absence of counts from this sample in SGF test was probably due to neither release of dead cells nor absence of bifidobacteria in the

interpolymer matrix, but due to the pH-dependent swellability of the matrix. The release of viable bifidobacteria cells from the interpolymer complex is desirable as probiotic microorganisms should not only be protected during upper gastrointestinal transit but the encapsulating matrix must also release live metabolically active cells into the intestines (Siuta-Cruz and Goulet, 2001).

The number of viable bacteria released from PEO-PPO-PEO:PVP:VA-CA matrix increased from 3.96 x 10^5 cfu/g to 9.03 x 10^7 cfu/g and then remained almost constant throughout 24 h of exposure (Fig. 4.1). At the end of 24 h of exposure, viable bifidobacteria counts were higher from PVP:VA-CA matrix when compared to non-encapsulated and those from PEO-PPO-PEO:PVP:VA-CA matrix (Fig. 4.1). PVP:VA-CA, our standard encapsulation system, completely protected the bacteria during exposure to SGF. An increase in the numbers of viable bacteria released from the interpolymer complex indicated efficient release properties of the complex at higher pH values. This meant that PVP:VA-CA has the desired properties, being insoluble at low pH thereby providing a protective coat for the bacteria against the detrimental acidity of the gastric fluid and opening up to liberate viable cells which will then colonize the intestinal epithelium and hence confer the purported beneficial effects to the host. The PEO-PPO-PEO:PVP:VA-CA matrix on the other hand did not protect the encapsulated bacteria from gastric acidity to the same extent. Some of the bacteria might have been released into and killed by the gastric fluid acidity.

4.4.2 Survival of bacteria in polycaprolactone (PCL)

Protection of encapsulated bifidobacteria by the standard system, PVP:VA-CA was compared with that of PCL, which also forms an interpolymer complex with VA-CA and is not hygroscopic. It was envisaged that the non-hygroscopic nature of PCL would minimize swellability of the interpolymer complex and thus protect the encapsulated bacteria in the simulated gastrointestinal fluids even better. The total number of *B. longum* Bb-46 cells encapsulated was determined in order to compare it with the total viable cells that are present at the end of exposure period. This would indicate how many

of the encapsulated cells were released and killed during exposure and how many were retained and protected.

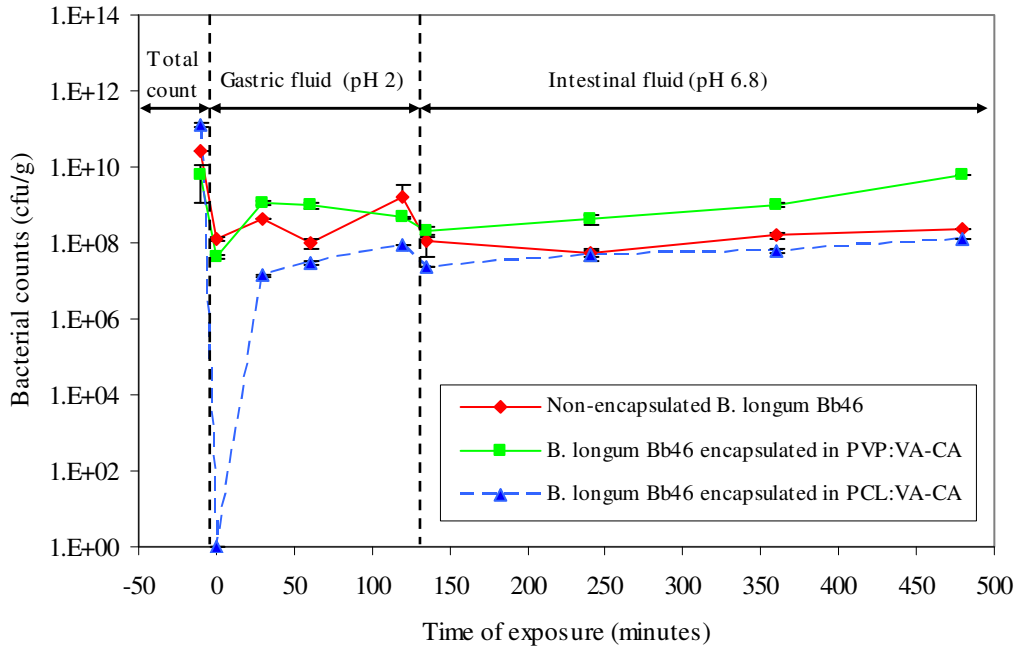


Figure 4.2: Viability of *B. longum* Bb-46 cells encapsulated in PCL:VA-CA and PVP:VA-CA after exposure to SGF and SIF

The total count of non-encapsulated bacteria was 2.7 x 10^{10} cfu/g while encapsulated bacteria were 6.4 x 10^{9} cfu/g and 1.4 x 10^{11} cfu/g for PVP:VA-CA and PCL:VA-CA interpolymer complexes, respectively (Fig. 4.2). The non-encapsulated bacteria count immediately upon suspension in SGF was 1.3 x 10^{8} cfu/g (Fig. 4.2). Numbers of non-encapsulated bifidobacteria fluctuated during exposure to SGF, with a count of 2.3 x 10^{8} cfu/g at the end of the exposure period (Fig. 4.2). The count for bacteria released from PVP:VA-CA encapsulation immediately upon suspension in SGF fluid was 4.2 x 10^{7} cfu/g, while no cells at this point were released from the PCL:VA-CA matrix (Fig. 4.2). The results obtained for PVP:VA-CA encapsulated cells highlighted the presence of batch to batch variations in the product produced using the same formulation at different times (under different conditions). The count of bacteria released from PVP:VA-CA matrix increased to 6.1 x 10^{9} cfu/g at the end of 2 h, while that of PCL:VA-CA

encapsulated bacteria increased from 0 to 1.4 x 10^8 cfu/g (Fig. 4.2). Release of bacteria from PCL:VA-CA encapsulation was delayed but once the matrix disintegrated it released encapsulated bacteria faster, exposing them to the low pH environment (Fig. 4.2). The delay in the release of encapsulated bacteria from the PCL:VA-CA interpolymer complex could be attributed to the hydrophobic nature of PCL (Pandey et al., 2005) causing slow absorption of the gastric fluid, though this desired effect was short lived, lasting only 30 min.

In the SIF a similar trend was observed for both encapsulation systems. Counts of released viable bacteria increased with exposure time (Fig. 4.2). Non-encapsulated bacteria numbers however increased from the initial count of 1.1 x 10^8 cfu/g immediately upon suspension in SIF to 2.3 x 10^8 cfu/g after 6 h, while those of encapsulated bacteria increased from 2.0 x 10^8 cfu/g and 2.4 x 10^7 cfu/g to 6.1 x 10^9 cfu/g and 1.4 x 10^8 cfu/g at the end of 6 h for PVP:VA-CA and PCL:VA-CA encapsulation matrices, respectively (Fig. 4.2). Viable counts from PCL:VA-CA encapsulation at the end of exposure period were lower than the non-encapsulated bacteria, whose count was lower than that of bacteria released from PVP:VA-CA (Fig. 4.2). Even though resistance of the PVP:VA-CA to SGF seemed reduced, this formulation still gave better protection and release of bacteria for colonization than when PCL was used. PCL seems to have only protected cells from early contact with SGF. Incorporation of PEO-PPO-PEO into this formulation also did not improve the properties of the matrix. PCL therefore seems to be a less suitable alternative than PVP even though it is non-hygroscopic.

4.4.3. Effect of GMS incorporation on survival in simulated gastrointestinal fluids

GMS is an acid stable, digestible flow modifier with good moisture and oxygen resistance and thus its inclusion as one of the ingredients for encapsulation may increase the survival of encapsulated probiotic cultures in gastric acid. In this study 8 % and 60 % GMS were included as one of the components of the interpolymer complex.

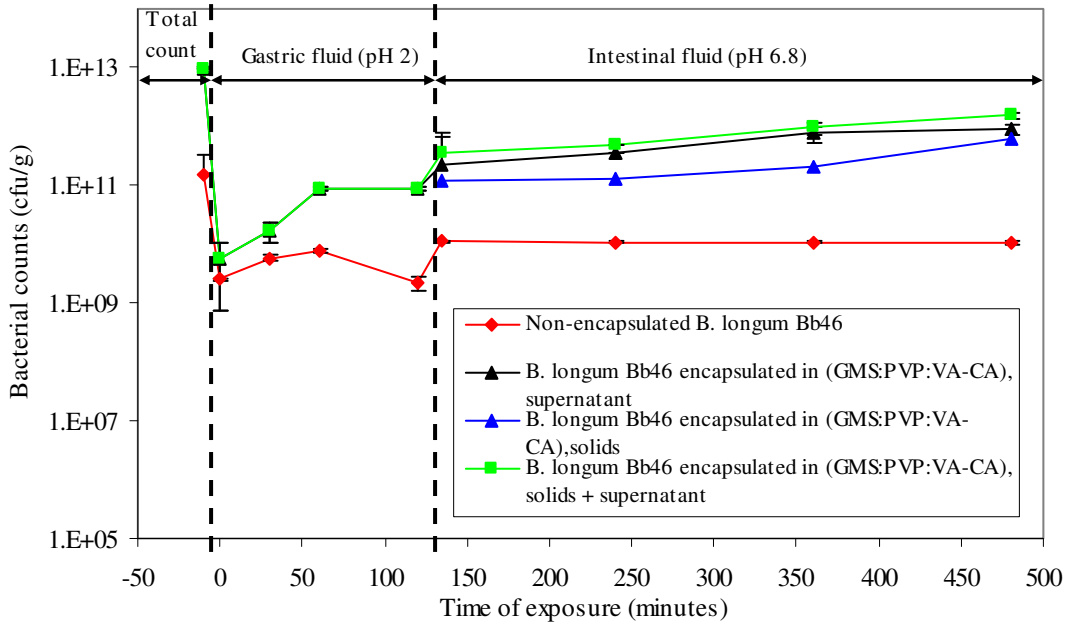


Figure 4.3: Effect of GMS incorporation into the interpolymer complex on survival of *B. longum* Bb-46 after exposure to SGF and SIF

The total (initial) counts for non-encapsulated bacteria and for bacteria encapsulated within the GMS:PVP:VA-CA encapsulated matrix were 1.5 x 10^{11} and 9.9 x 10^{12} cfu/g, respectively (Fig. 4.3). Immediately upon suspension in SGF counts of non-encapsulated bacteria decreased to 2.5 x 10^{9} cfu/g (Fig. 4.3). The count decreased to 2.3 x 10^{9} cfu/g at the end of the 2 h of exposure (Fig. 4.3). The reduction of non-encapsulated bacteria was once again minimal as was observed in our earlier trials. The initial count of 5.6 x 10^{9} cfu/g for GMS:PVP:VA-CA encapsulated bacteria immediately upon suspension in SGF increased to 9.0 x 10^{10} cfu/g at the end of the 2 h (Fig. 4.3). At this point the count for encapsulated bacteria was already higher than the non-encapsulated bacteria (Fig. 4.3).

The SIF counts of non-encapsulated bacteria decreased from 1.12 x 10^{10} cfu/g to 1.07 x 10^{10} cfu/g after 6 h of exposure (Fig. 4.3). In order to determine how many of the bifidobacteria already released into the SGF would survive the SIF (pH 6.8), the supernatant from the gastric fluid trial was analyzed. The solid fraction was analyzed to see further release of live bacteria from the interpolymer matrix when the pH of the

environment was increased. Interestingly, an increase in counts was observed for both the supernatant and the solids in SIF (pH 6.8) (Fig. 4.3). The overall count for live bifidobacteria released from the GMS:PVP:VA-CA matrix increased from 3.5 x 10^{11} cfu/g to 1.5 x 10^{12} cfu/g at the end of the 6h (Fig. 4.3). An increase in the counts from the supernatant indicated the possibility that some of the cells released were still in clumps which were breaking up in SIF or held together by some remnants of the interpolymer complex, which also swelled and disintegrated further in the SIF to liberate single cells. An increase in number from the solids indicated that even though some bacteria were released into the SGF most of the bifidobacteria were still retained and protected inside the interpolymer matrix. An increase in numbers indicates swelling and release from the solid matrix in the SIF. Comparing the total encapsulated bacteria and the final viable count it was observed that most of the encapsulated bacteria were protected and therefore survived exposure to SGF (pH 2) and were released in SIF (pH 6.8) (Fig. 4.3). Therefore, GMS:PVP:VA-CA matrix provided protection for encapsulated bacteria when compared to non-encapsulated bacteria.

4.4.4 Effect of a higher GMS loading on protective properties of the GMS:PVP:VA-CA interpolymer matrix

The GMS loading in the interpolymer complex was increased from 8% to 60% in an attempt to improve the protection afforded by the interpolymer complex. Total viable cell numbers were 4.8 x 10^{12} cfu/g and 2.9 x 10^{12} cfu/g for the non-encapsulated and encapsulated bacteria respectively (Fig. 4.4). The behaviour of the interpolymer complex in SGF was still the same as when lower GMS was used, resulting in release of some of the encapsulated cells into the lower pH environment. Immediately upon suspension of GMS:PVP:VA-CA encapsulated material with higher loading of GMS, in SGF 3.5 x 10^{8} cfu/g were released (Fig. 4.4). This count increased to 1.4 x 10^{11} cfu/g at the end of 2 h of exposure to SGF (Fig. 4.4). On the other hand, counts of non-encapsulated bacteria increased slightly from an initial count of 2.0 x 10^{11} cfu/g to 2.1 x 10^{11} cfu/g at the end of 2 h (Fig. 4.4).

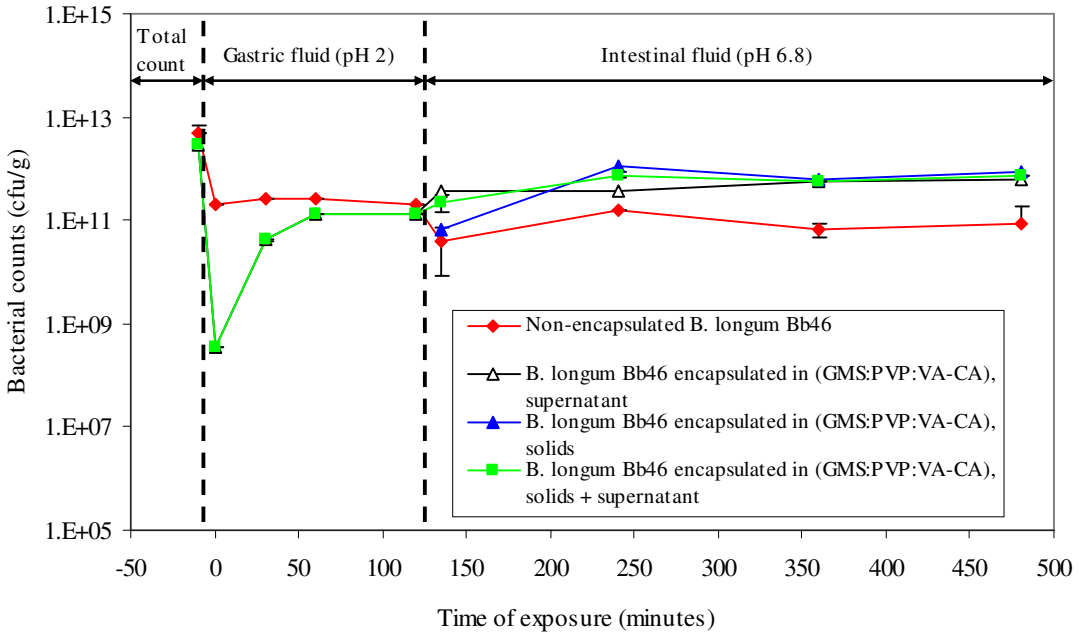


Figure 4.4: Effect of high GMS loading on survival of *B. longum* Bb-46 after exposure to SGF and SIF

In the SIF (pH 6.8), numbers of non-encapsulated bacteria increased from 4.1 x 10^{10} cfu/g immediately upon exposure to 8.4 x 10^{10} cfu/g at the end of 6 h (Fig. 4.4). The increase in numbers of non-encapsulated bacteria could not be explained, but may be due to growth of these bacteria in the SIF. Encapsulated bacteria released from the interpolymer matrix increased from 3.9 x 10^{11} cfu/g to 7.4 x 10^{11} cfu/g at the end of 6 h (Fig. 4.4). When comparing the final values for viable encapsulated bacteria with the total initial values of encapsulated bacteria, it was observed that the total number of viable cells released from the complex with 60 % GMS after 6 h of exposure to SIF was higher than that for interpolymer complex with 8 % GMS. Thus, more of the encapsulated cells were released and killed in the gastrointestinal fluids when 8 % GMS was incorporated in the interpolymer complex than when 60 % was used. Higher loading of GMS therefore improved the protection efficiency of the interpolymer complex.

4.4.5 Effect of gelatine capsule on survival of GMS:PVP:VA-CA encapsulated bacteria in simulated gastrointestinal fluids

Gelatine capsules are one of the generally accepted dosage forms for delivery of probiotics via the oral route and have been used for administration of probiotics (Saxelin et al., 1995). The effect of 8 % GMS encapsulation on bacterial survival was, in this case, coupled with enclosure of encapsulated bacteria within gelatine capsules. No bacteria were released from the gelatine capsule immediately upon exposure of the capsule to SGF (pH 2) for both non-encapsulated and encapsulated bacteria (Fig. 4.5). The gelatine capsule did not dissolve immediately, but only dissolved to release the non-encapsulated bacteria after about 30 min, while the encapsulated bacteria were detected only after 1 h of exposure (Fig. 4.5). A count of 1.1 x 10^7 cfu/g was obtained for non-encapsulated bacteria after 30 min, which increased after 1 h to 3.7 x 10^9 cfu/g as the gelatine capsules dissolved completely releasing more of the bacteria into the SGF (Fig. 4.5).

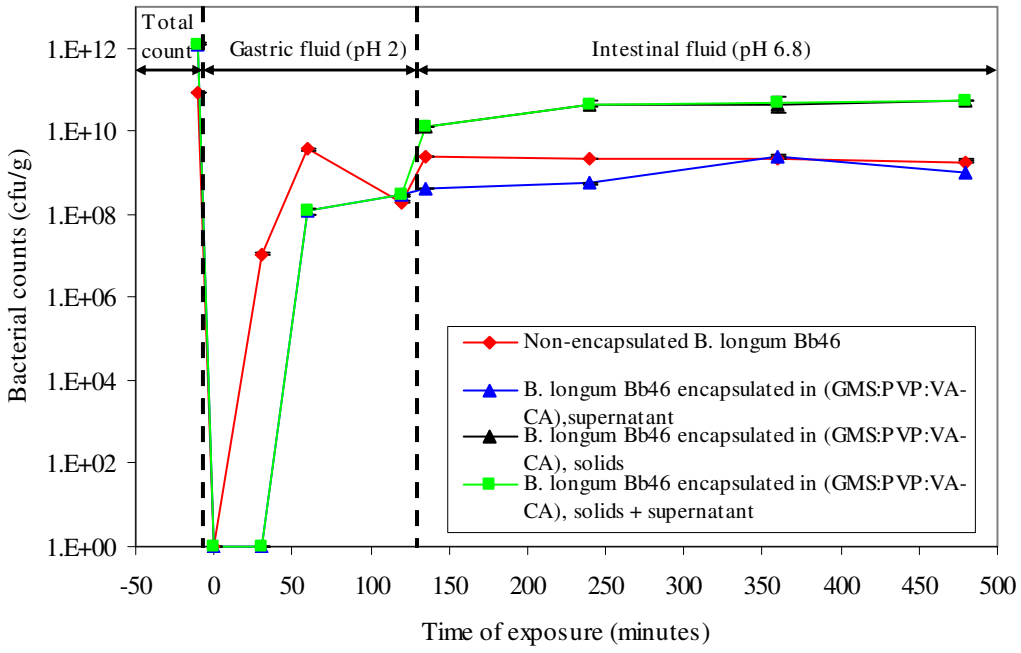


Figure 4.5: Effect of enclosure of GMS:PVP:VA-CA encapsulated *B. longum* Bb-46 into gelatine capsules on survival after exposure to SGF and SIF

The count for non-encapsulated bacteria then decreased to 1.9 x 10^9 cfu/g after 2 h of exposure (Fig. 4.5), indicating death of the cells once they were suspended in the acidic SGF. Encapsulated bacteria were only released into the SGF after 1 h with a count of 1.2 x 10^8 cfu/g (Fig. 4.5). After 2 h the count increased to 2.9 x 10^8 cfu/g (Fig. 4.5). Thus the gelatine capsules delayed contact between gastric acidity and the bacteria. This delay was more effective in the case of encapsulated bacteria, as it was coupled with that of the interpolymer complex within which bacteria were protected. That is, in the case of encapsulated bacteria, the gelatine capsule served as a second layer of protection to the bacteria. Comparing the numbers of bacteria released at the end of exposure to SGF from GMS:PVP:VA-CA analyzed as free powder (Fig. 4.3) and when enclosed into gelatine capsules (Fig. 4.5), it was evident that the gelatine capsules had an added advantage as fewer cells were released when the capsules were present, 2.9 x 10^8 cfu/g, compared to 9.0 x 10^{10} cfu/g for free powder. Thus, the gelatine capsule minimized the number of bacteria released into the detrimental acidic gastric fluid.

In the SIF, counts of non-encapsulated bacteria decreased over the 6 h, and were lower than counts from the solid portion of encapsulated material (Fig. 4.5). Encapsulated bacteria from the supernatant increased over the first 4 h into the SIF, probably due to further release from the polymer matrix (Fig. 4.5). However their numbers decreased after 6 h, which indicated death of some of the released cells (Fig. 4.5). Bacteria released from the solid portion increased throughout 6 h from 1.3 x 10^{10} cfu/g to 5.4 x 10^{10} cfu/g (Fig. 4.5). The final count of viable bifidobacteria released from the GMS:PVP:VA-CA in capsules was higher that the non-encapsulated bacteria count (Fig. 4.5). This indicated that gelatine capsules protected bacteria from the detrimental conditions to which bacteria were exposed.

4.4.6 Effect of beeswax on survival in simulated gastrointestinal fluids

Beeswax is a food grade material with about 20 % free acid groups which can form a complex with the basic groups of PVP, and also has very good acid and moisture resistance. These properties could potentially improve survival of encapsulated bacteria when beeswax is used as one of the ingredients in encapsulation. Rao et al. (1989) coated microspheres containing *B. pseudolongum* with 1 % beeswax and these coated microspheres exhibited the highest survival of *B. pseudolongum* after sequential incubation in SGF for 30 min followed by SIF. In this study, it was observed that high numbers of encapsulated bacteria were released from the beeswax:PVP encapsulated material immediately upon suspension of this material in SGF (Fig. 4.6).

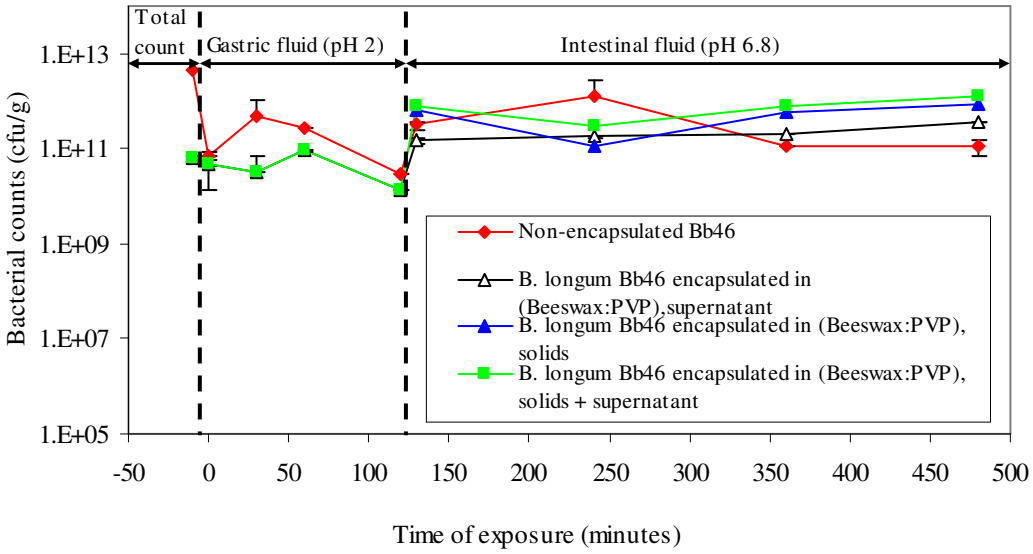


Figure 4.6: Survival of *B. longum* Bb-46 encapsulated in beeswax:PVP after exposure to SGF and SIF

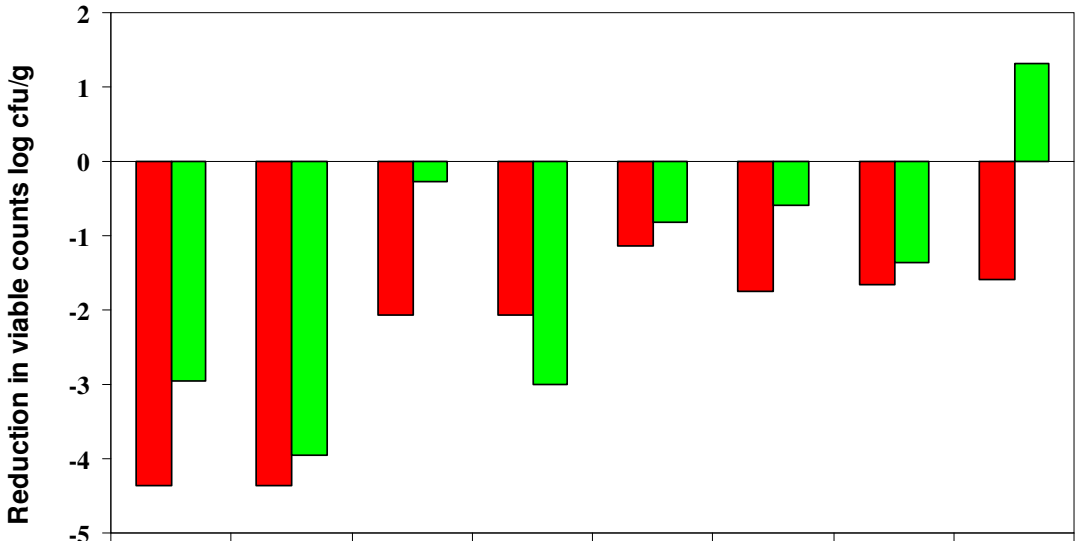
This could be due to insufficient compatibility between beeswax and PVP leading to insufficient interpolymer complex strength and/or faster release. The numbers increased over 2 h from 6.1 x 10^{10} to 1.4 x 10^{11} cfu/g (Fig. 4.6). Non-encapsulated bacteria decreased from an initial count of 1.4 x 10^{11} to 3.0 x 10^{10} cfu/g after 2 h of exposure to

SGF (Fig. 4.6). Encapsulated bacteria counts were higher than the non-encapsulated bacteria counts at the end of exposure to SGF (Fig. 4.6).

In the SIF, counts of non-encapsulated bacteria decreased to 1.12 x 10^{11} cfu/g at the end of the 6 h, while counts of encapsulated bacteria increased to 1.07 x 10^{12} cfu/g (Fig. 4.6). That is, at the end of exposure, the total number of *B. longum* Bb-46 cells released from the beeswax:PVP encapsulated material was higher than the total count of bifidobacteria reported to be encapsulated in this interpolymer complex. This was probably due to incomplete release during initial counts whereby release of bacteria from the interpolymer complex was done in Ringer's solution (pH 6.8). Beeswax is insoluble in Ringer's solution but it is digestible. Therefore use of Ringer's solution led to an underestimation of the total encapsulated bacteria. Complete release of bacteria from the beeswax:PVP interpolymer complex would have been obtained if SIF was used for release.

4.4.7 Comparing protection efficiencies of the different formulations

Reduction in numbers of viable bacteria at the end of the experimental period was always higher for non-encapsulated than for encapsulated bacteria regardless of the interpolymer complex formulation used, except when PCL:VA-CA was used (Fig. 4.7). Reduction in the numbers of viable cells encapsulated in the PVP:VA-CA interpolymer complex, our normal system, was not the same for products from different batches. The encapsulated bacteria from this interpolymer complex were reduced by 0.28 log cfu/g in one batch and 2.96 log cfu/g in the other (Fig. 4.7). This pointed towards some batch-to-batch variations in the protection and release efficiency of the same formulation, which must still be looked into. When comparing the highest loss of cells from the normal system with other formulations tested, it was observed that incorporation of GMS and use of gelatine capsules improved protection efficiency of the normal system (Fig. 4.7).



	Normal system	Normal system + PEO-PPO-PEO	Normal system	PCL:VA-CA	Normal system + GMS (8%)	Normal system + GMS (60%)	Normal system + GMS + gelatin	Beeswax:PVP
Non-encapsulated	-4.36	-4.36	-2.07	-2.07	-1.14	-1.76	-1.67	-1.6
Encapsulated	-2.96	-3.95	-0.28	-3	-0.81	-0.59	-1.36	1.31

Figure 4.7: Summary of reductions in viable counts of non-encapsulated and encapsulated bacteria over the experimental period

GMS alone resulted in lower reduction when compared to gelatine capsules (Fig. 4.7). Higher loading of GMS improved the protection efficiency further (Fig. 4.7). There was higher loss of viable cells from the PCL:VA-CA and basic system with PEO-PPO-PEO than for non-encapsulated bacteria. This indicated that incorporation of PEO-PPO-PEO into the encapsulation matrix and use of polycaprolactone had negative effects on the release properties and hence protection efficiency of the interpolymer complex. When beeswax was used there was no loss of cells observed at the end as was with other formulations. Final viable counts of bacteria released from the beeswax:PVP matrix were higher than the initial counts. This indicated that there was incomplete release of bacteria from the interpolymer complex when initial counts were determined because of the use of Ringer's solution instead of SIF.

4.4.8 The effect of encapsulated *B. longum* Bb-46 on the microbial community of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) model

This part of the research was carried out by researchers at the Laboratory of Microbial Ecology and Technology, Ghent University, Belgium. Their laboratory is equipped with a dynamic model of the human gastrointestinal tract termed Simulator of the Human Intestinal Microbial Ecosystem (SHIME). The model has vessels simulating different compartments of the gastrointestinal tract, with the temperature maintained at 37^{o}C. This model has been used for *in vitro* monitoring of the microbial community of the gastrointestinal tract (Alander et al., 1999, Van de Wiele et al., 2004). Similar models were used for the same purpose by researchers elsewhere (Marteau et al., 1997). Their results are included in this thesis to substantiate the findings reported in this chapter. The aim of their study was to evaluate survival of *B. longum* Bb-46 in the different vessels of the model and to further investigate to what extent this bacterium establishes or maintain itself among other members of the large intestine microbial community.

The SHIME system was inoculated with fecal isolates and these were allowed 3 weeks to colonize different compartments of the model. Microbial communities characteristic to the microbial communities found in the colon compartments *in vivo* developed during this period. These were used to monitor the baseline levels of fermentation activity and microbial community in different vessels. Then the SHIME was inoculated with 1 g non-encapsulated *B. longum* Bb-46 everyday for a week followed by a washout period of a week. After this, PVP:VA-CA encapsulated *B. longum* Bb-46 were introduced, also 1g everyday for a week, followed by a washout period. The microbial community was analyzed using plate counts, Polymerase-Chain reaction – Denaturing Gradient gel electrophoresis (PCR-DGGE) and real time-PCR.

It was observed that both the non-encapsulated and encapsulated *B. longum* Bb-46 survived in the stomach and small intestine vessels. Interestingly, as was observed in our laboratory, survival was better for encapsulated than for non-encapsulated bacteria. Results also indicated that non-encapsulated bacteria had a chance of reaching the colon

environment in relatively high concentrations. This was observed whereby numbers of non-encapsulating bacteria, though they decreased upon exposure to simulated gastrointestinal fluids, were not reduced to levels lower than the recommended minimum for beneficial effects of probiotics cultures. Very important though is that encapsulated bacteria survival surpassed that of non-encapsulated bacteria by several orders of magnitude. PCR-DGGE, though it detects both viable and non-viable members of the population, showed that the population of bifidobacteria in the SHIME model was increased by introduction of *B. longum* Bb-46. A real-time PCR method that will quantify the specific *B. longum* strain added to the SHIME model is still under development. A report with all the results from the SHIME trials is included as an Appendix to this thesis.

4.5 CONCLUSIONS

Encapsulation of probiotics in an interpolymer complex in $scCO_2$ has potential for their protection from the detrimental effects of gastric fluid, and release in the intestines, where the released cells can then bind to the epithelium and colonize. PVP:VA-CA interpolymer complex, our standard system, protected *B. longum* Bb-46 from the gastric juice and then released cells in the SIF fluid for colonization. The presence of glyceryl monostearate as one of the ingredients for encapsulating *B. longum* Bb-46 improved the protection efficiency of the PVP:VA-CA complex with increased protection when a higher loading of GMS was incorporated. Use of an alternative hydrophobic polymer (PCL) and ethylene oxide-propylene oxide triblock copolymer (PEO-PPO-PEO) did not provide protection for encapsulated bacteria. There were batch to batch variations in final products using the same formulation, which needs to be looked into as products of good quality should be consistent. Encapsulation of more sensitive strains of bifidobacteria with this system could offer even more significant protection when compared to non-encapsulated cells.

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CHAPTER 5

Shelf life studies of *Bifidobacterium lactis* Bb-12 encapsulated in interpolymer complexes in supercritical CO_2 stored under different storage conditions

5.1 ABSTRACT

A reduction in numbers of viable probiotic cultures in products results in lower than recommended levels for beneficial effects of these organisms in the host. Several attempts for improving survival of probiotics in products including encapsulation in different materials have been tried. However, none of the reported methods of encapsulation investigated the effect of encapsulation of probiotics in polymers in a supercritical environment. This study investigated the effect of interpolymer complex formed in $scCO_2$ on the shelf life of *Bifidobacterium* lactis Bb-12 under different storage conditions. The protection efficiencies of different polymer formulations were compared. These included PVP:VA-CA, PCL:VA-CA, PVP:VA-CA with GMS, addition of poly(ethylene oxide-*co*-propylene oxide) or PEO-PPO. Non-encapsulated and encapsulated bacteria were stored in either sterile plastic bags or glass bottles at 4 ^{o}C, 30 ^{o}C, 40 ^{o}C, 30 ^{o}C with 60 % relative humidity and room temperature. Samples were taken over time and analysed for viable bacteria. PVP:VA-CA encapsulated bacteria survived better than non-encapsulated bacteria under all storage conditions when the product was recovered from the reaction chamber. When the product was recovered from the product chamber, numbers of viable non-encapsulated bacteria were higher than the encapsulated bacteria numbers for all interpolymer complex formulations. However, it was later realized that increased release times (6 h instead of 2 h) are required to more accurately determine the total viable bacteria present in the encapsulation matrix. When these conditions were used the encapsulated probiotics always had improved shelf life compared to the non-encapsulated bacteria. The PCL:VA-CA interpolymer complex seemed weaker than the PVP:VA-CA interpolymer complex as viable counts of bacteria released from it were lower than those from the latter complex. Inclusion of PEO:PPO to both complexes had a negative effect on the protection efficiency of the matrices. Survival of bacteria was better at lower temperatures than at higher temperatures, with the humidity chamber being the most unfavourable conditions for storage.

Keywords: *Bifidobacterium lactis* Bb-12, supercritical CO_2, polycaprolactone, Glyceryl monostearate, polyethylene oxide-propylene oxide copolymer, encapsulation

5.2 INTRODUCTION

A normal distribution of the intestinal microflora can be maintained by addition of bifidobacteria and bifidogenic products to diet (Rao et al., 1989). Health benefits can be obtained through consumption of milk products such as cheese and yoghurt, and pharmaceutical preparations containing bifidobacteria and lactobacilli (Hsiao et al., 2004). Bifidobacteria have low acid tolerance and as a result some are often killed by the acidity of yoghurt decreasing their viable numbers (Sun and Griffiths, 2000). To confer beneficial effects in the host, bifidobacteria not only have to survive production processes, but they must also survive in the product, the upper gastrointestinal tract and in the food vehicle during its shelf life (Sun and Griffiths, 2000; Jayamanne and Adams, 2004).

A decline in viable counts of *L. acidophilus* and *Bifidobacterium* spp was reported by several market surveys on probiotic products from different countries aroud the world including Argentina, Australia, North America and South Africa. Cultures in products were present in levels much lower than the recommended levels at the end of shelf storage (Micanel et al., 1997, Shah et al., 2000, Vinderola et al., 2000, Elliot and Teversham, 2004; Huff, 2004,). This indicates clearly that shelf life stability of probiotics in food and pharmaceutical products is still a worldwide problem.

It was shown in the recent study that successful encapsulation of bifidobacteria in an interpolymer complex formed between PVP and VA-CA was achieved (Chapter 3). Also, it was indicated that the interpolymer complex protected the encapsulated bacteria from the detrimental conditions of the upper gastrointestinal tract, releasing few or no cells in the simulated gastric fluid and then releasing more cells at the higher pH environment of the simulated intestinal fluid (Chapter 4). Protection of probiotic bacteria from the gastric acidity is very important for their efficiency. Maintenance of probiotic cultures' viability in products until their consumption in order to ensure the delivery of purported health benefits has been of much interest (Akalin et al., 2004). It is therefore important that the encapsulation method provides protection to bacteria in the products,

keeping them viable throughout the shelf life ensuring that consumers receive the health benefits from the ingested probiotics.

Development of a method to improve survival of probiotics in commercial products is much needed and will benefit both suppliers and consumers. This study investigated effects of encapsulation using encapsulation with an interpolymer complex in $scCO_2$ on survival of probiotic *B. lactis* Bb-12 under different storage conditions.

5.3 MATERIALS AND METHODS

5.3.1 Encapsulation of bacteria

Encapsulation of bacteria was done according to Moolman et al. (2006) with modifications in the mixing reactor procedure. In the mixing reactor procedure, after 2 h of equilibration with intermittent stirring the reactor was depressurized and the solid polymer-probiotic mixture was then removed and ground to a fine powder using a coffee grinder.

5.3.2 Storage of samples

Non-encapsulated and encapsulated bacteria were stored in either sterile plastic bags or sterile glass bottles. Containers with bacteria were stored at different conditions (room temperature, 4 ^{o}C, 40 ^{o}C, 30 ^{o}C, 30 ^{o}C with 60 % relative humidity) for the duration of the experiments. 1 g samples were taken from each container on analysis dates for the enumeration of viable bacteria.

5.3.3 Enumeration of bacteria

Two test tubes each containing 9 ml of sterile ¼ strength Ringer's solution adjusted to pH 6.8 were prepared. 1 g of encapsulated and non-encapsulated bacteria were added to separate tubes and the tubes were incubated anaerobically at 37 ^{o}C for 2 h to allow

release of encapsulated bacteria from the interpolymer complex matrix. Serial dilutions of the suspension were made in sterile ¼ strength Ringer's solution. 0.1 ml of appropriate dilutions was pour plated in triplicate onto MRS agar supplemented with 0.05 % cysteine hydrochloride. The plates were incubated anaerobically in anaerobic jars with Anaerocult A gaspaks (Merck), at 37 ^{o}C for 72 h. Anaerocult C test strips were used for indication of anaerobic conditions inside the jars. The numbers of colonies grown were counted and from these the numbers of viable cells were calculated (cfu/g).

5.4 RESULTS AND DISCUSSION

5.4.1 Survival of *B. lactis* Bb-12 encapsulated in PVP:VA-CA interpolymer complex (normal system) under different storage conditions

Since to our knowledge this work was the first to report the formation of an interpolymer complex in $scCO_2$ for encapsulation of probiotics, there were no similar results for comparison. However, comparison of results obtained was done with the results of studies in which probiotics were encapsulated using any other methods reported in literature. Also, most of the studies, summarized by Champagne and Gardner, 2005, investigated survival of probiotics either in milk or yoghurt (or other fermented dairy products), not when encapsulated probiotics were stored as powder as was the case in the recent study. Low levels of bifidobacteria in commercial products have been correlated with presence of starter cultures in products (Champagne and Gardner, 2005). Interference by starters has been excluded by analysing products containing only the incorporated probiotic culture.

Most of the published results on shelf life of probiotics have been done on products stored under refrigerated conditions (Shah et al., 1995; Adhikari 2000; Vinderola et al., 2000; Hansen et al., 2002). We compared survival of bacteria stored at room temperature, 4 ^{o}C and 30 ^{o}C with a view that should encapsulation improve survival of probiotic bacteria at higher temperatures such as room temperature and 30 ^{o}C then the problem of limitation of probiotic use to communities with limited access to refrigeration

would be alleviated. Hence, benefits of probiotic use could be made available to the community at large, including underprivileged communities without electricity. Secondly, the effects of unfavourable conditions associated with long distance transport, compromising stability of probiotics e.g. in pharmaceutical preparations, could also be minimised.

Numbers of viable *B. lactis* Bb-12 decreased during all storage conditions for both encapsulated and non-encapsulated bacteria. The encapsulated bacteria indicated in both Fig. 5.1 and 5.2 were harvested from the mixing reactor. There was an increase in the numbers of viable bacteria for all the samples except non-encapsulated bacteria stored at room temperature, after 6 weeks of storage (Fig. 5.1). The increase in the numbers could not be explained. However, Saxellin et al., 1999 reported that in some instances where numbers of probiotic bacteria increased during storage, this increase was related to the splitting of the bacteria from chains into single cells. Numbers of viable bacteria released from the PVP:VA-CA interpolymer complex were lower than those of non-encapsulated bacteria under both storage conditions (Fig. 5.1).

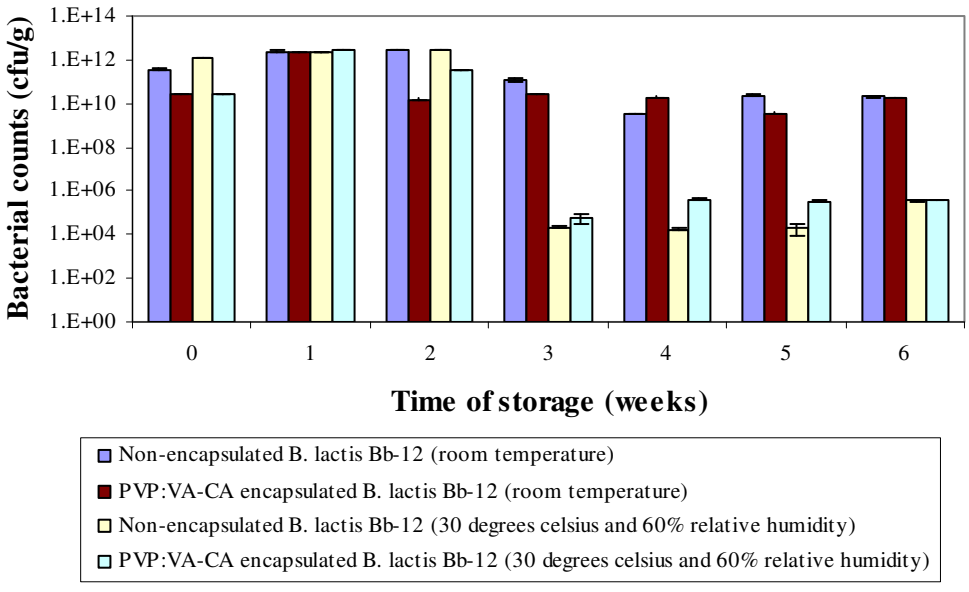

Figure 5.1: Survival of *B. lactis* Bb-12 during storage at room temperature and at 30 ^{o}C and 60 % relative humidity

After seven weeks of storage at room temperature counts of non-encapsulated bacteria decreased from 10^{11} cfu/g to 10^{7} cfu/g while counts of viable bacteria released from the interpolymer complex decreased from 10^{12} cfu/g to 10^{10} cfu/g (Fig. 5.2). At 30 ^{o}C / 60 % RH counts of non-encapsulated bacteria decreased to 10^{5} cfu/g after 6 weeks while viable counts from the interpolymer matrix decreased to 10^{7} cfu/g. The decrease in number of viable bacteria during the first 3 weeks of storage was more rapid for bacteria stored in the humidity chamber than those at room temperature. Adaptation of survivors under these conditions resulted in a steady decline in numbers in the later stages of storage. Storage temperature is among factors affecting viability of probiotic bacteria (Dave and Shah, 1997; Kailasapathy and Rybka, 1997). Bacteria survived better at lower storage temperature. Results were in agreement with those of Wang et al. (2004) who found that survival of test organisms in dried fermented soymilk was better at 4 ^{o}C than at 25 ^{o}C. The encapsulated bacteria were 3 logs higher than non-encapsulated in the humidity chamber and 1-2 logs higher at room temperature. This indicates that encapsulation provided some protection to the encapsulated cells.

Comparing the two encapsulated bacteria under the two test conditions it was observed that the bacteria in the drawer survived better than those at 60 % relative humidity (Fig. 5.1). High temperature and relative humidity were more detrimental to the cells when compared to room temperature. The advantage of the interpolymer complex was more pronounced under more unfavourable conditions. After 3 weeks of storage at 30 ^{o}C/60 % RH, counts of non-encapsulated bacteria dropped to levels lower than the recommended minimum for beneficial effects of probiotics while those of encapsulated bacteria remained above this minimum. Interpolymer complex therefore increased the shelf life under unfavourable conditions from 3 to 6 weeks. The advantage of encapsulation was not as pronounced at room temperature as the levels of non-encapsulated bacteria did not drop below the recommended minimum, but better survival would allow lower loading levels in the final product.

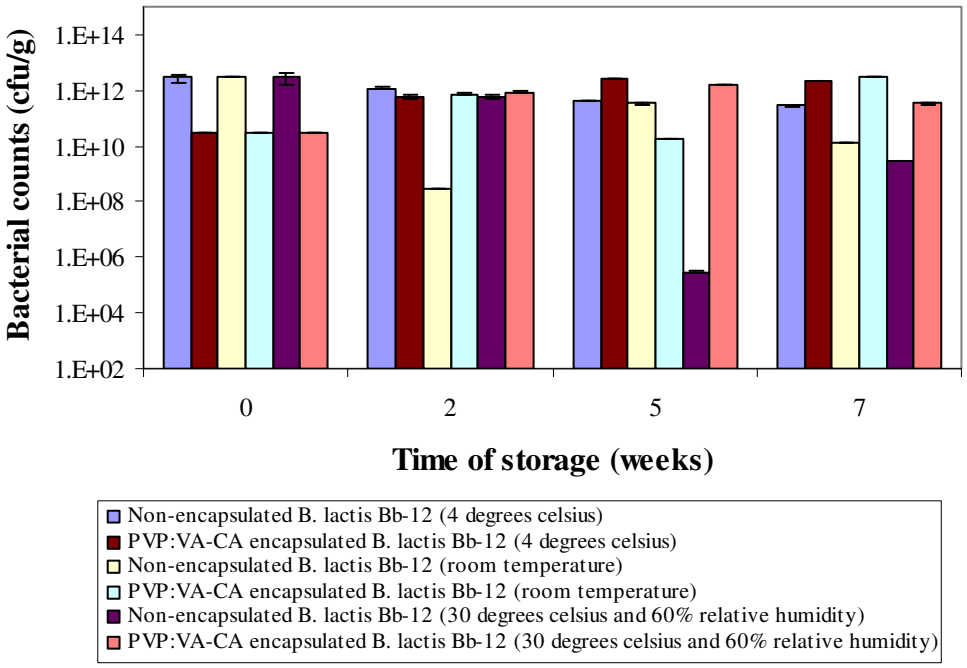

Figure 5. 2: Survival of *B. lactis* Bb-12 under different storage conditions

When an additional storage temperature (4 ^{o}C) was included a similar trend was observed whereby survival was better at this lower temperature of storage than at high temperature conditions tested (Fig. 5.2). Thus, bacteria at 4 ^{o}C survived better than those at room temperature and at 30 ^{o}C/ 60 % RH. Numbers of viable bacteria released from the interpolymer complex were higher than counts of non-encapsulated bacteria under all tested storage conditions. Overall survival was 4 ^{o}C > room temperature > 30 ^{o}C/ 60 % RH. Researchers elsewhere observed similar results whereby better survival of probiotic cultures during storage was at low than at high temperatures (Sun and Griffiths, 2000; Hsiao et al., 2004). Encapsulation increased survival of *B. lactis* Bb-12 indicating the potential for PVP:VA-CA interpolymer complex encapsulation to increase the shelf life of probiotic cultures.

5.4.2 Comparison of survival of *B. lactis* Bb-12 encapsulated in PVP:VA-CA (normal system) and PCL:VA-CA interpolymer complexes during storage at room temperature

The encapsulated bacteria in this case and all the subsequent results in this chapter were recovered from the product chamber. The PVP:VA-CA interpolymer complex protected *B. lactis* during storage. The protection efficiency of this basic system was compared to that of PCL:VA-CA, which was shown to delay contact between encapsulated bacteria and the gastric acidity. Numbers of viable bacteria decreased over the experimental period. Non-encapsulated bacteria had higher numbers of viable bacteria than encapsulated bacteria from both formulations (Fig. 5.3). High viable counts for non-encapsulated bacteria might have been due to intrinsic resistance of *B. lactis* Bb-12 to unfavourable conditions while low viable counts for encapsulated bacteria could probably be due to incomplete release of encapsulated bacteria from the interpolymer complex within the 2 h of incubation in Ringer's solution allowed for release. However, earlier controlled release studies indicated that there was about 85 % release of encapsulated indomethacin using this method, after 24 h exposure to more alkaline environment (pH 6.8) with about 50 % release occurring in the first 3 h. This is due to the difference in sizes of bacteria (~1 μm) and indomethacin (~1 nm) resulting in faster release of the drug because of its smaller size, than the bacteria which are bigger and would therefore require swelling of the polymer matrix to a larger extent (thus for a longer period) before release of the bacteria can occur. The reduction in numbers of both non-encapsulated and encapsulated bacteria was not as sharp as was observed when samples were stored in sterile plastic bags.

Later in the thesis, chapter (6) we show the effect of release time on counts from the encapsulated system. It is clear from those results that release time before counts is a critical aspect of determining accurately the number of viable bacteria from the encapsulated system. The fact that analysis in this instance was done after only 2 h of release, explains the lower levels observed in this trial.

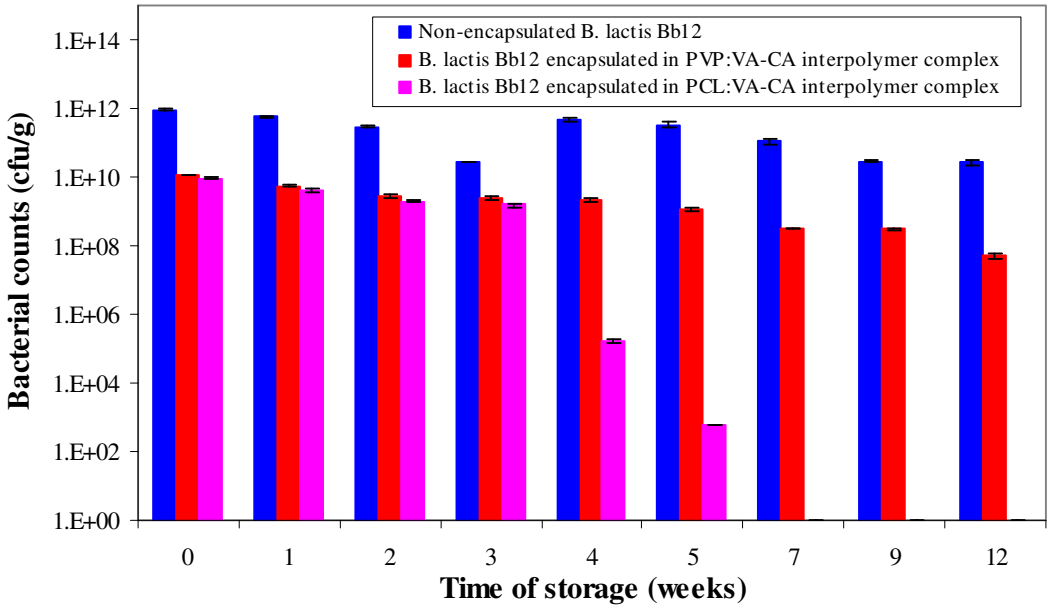

Figure 5.3: Viable counts of PVP:VA-CA and PCL:VA-CA encapsulated *B. lactis* Bb-12 over time

In this experiment samples were at room temperature in sterile polytop bottles. The slow reduction in numbers could therefore be due to storage of sample in glass bottles which minimized entry/diffusion of oxygen through the container. Researchers elsewhere found that bacteria stored in glass bottles survived better. Hsiao et al. (2004) and Dave and Shah (1997) found that survival of bifidobacteria was better when bacteria were stored in glass bottles than when stored in polyester bottles. High survival in glass bottles can be attributed to the relatively low oxygen permeability of glass bottles when compared to polyester bottles. Oxygen permeation through packaging was shown to negatively affect viability of bifidobacteria in milk and yoghurt (Ishibashi and Shikamura, 1993).

The numbers of viable bacteria released from both interpolymer complexes were lower than counts of non-encapsulated bacteria (Fig. 5.3). Throughout the storage period, levels of non-encapsulated bacteria remained above the recommended minimum for beneficial effects (Fig. 5.3). High stability or survival of *B. lactis* Bb-12 found in this study was in correlation with results observed by researchers elsewhere. Auty et al. (2001) found that *B. lactis* Bb-12 inoculated in cheddar cheese survived during ripening

at levels around 10^8 cfu/g. Lahtinen et al. (2003) observed that plate counts of *B. lactis* Bb-12 remained stable during storage in fermented oat products. The decrease in the numbers of viable bacteria released from PVP:VA-CA and PCL:VA-CA interpolymer complex matrices were similar during the first 4 weeks of storage (Fig. 5.3). Counts of viable bacteria released from PCL:VA-CA dropped to levels lower than 10^6 cfu/g after 6 weeks of storage and were undetectable after 7 weeks (Fig. 5.3). Viable counts for *B. lactis* Bb-12 from the PVP:VA-CA interpolymer complex matrix remained higher when compared to those from PCL:VA-CA even though levels for both matrices were lower than those of the non-encapsulated bacteria (Fig. 5.3). The number of bacteria in PVP-VA-CA remained high throughout the experimental period, giving counts of 3.23 x 10^9 cfu/g after 9 weeks (Fig. 5.3). Acceptable shelf life for encapsulated probiotics was 5 and 9 weeks for PCL-VA-CA and PVP-VA-CA encapsulations, respectively. The results indicated that PVP:VA-CA interpolymerx complex had better protection efficiency that PCL:VA-CA. The difference in survival of bifidobacteria when different polymers were used could be because of the differences in the chemical properties of the different polymers and how they react/change/behave in presence of $scCO_2$. The interpolymer complex formed between PCL and VA-CA seemed to be weaker than the PVP:VA-CA interpolymer complex, rendering it less protective for encapsulated bacteria. Higher numbers of non-encapsulated bacteria indicated that *B. lactis* Bb-12 was intrinsically resistant to detrimental conditions.

The other factor impacting on release from the encapsulation matrix is that it seems to agglomerate somewhat with time during storage (especially with exposure to humid conditions). These larger agglomerates seem to slow down release and thus the numbers of released bacteria after 2 h decrease with time for the encapsulated systems. When 6 h release time is used, this situation improves significantly (see Chapter 6). Six hours release time is not a major problem for the probiotic system if ultimately applied *in vivo*, as the main desired area of release and colonisation is in the colon, which is reached approximately 4-8 h after the particles leave the gastric environment.

5.4.3 Effect of PEO-PPO-PEO triblock copolymer inclusion on the protection efficiency of interpolymer complex matrices under different storage conditions

PEO-PPO was used to enhance flow in $scCO_2$ and aid in the formation of fine particles for encapsulated bacteria powder. When added to the PVP:VA-CA interpolymer complex, initial counts for encapsulated and non-encapsulated bacteria were the same (Fig. 5.4).

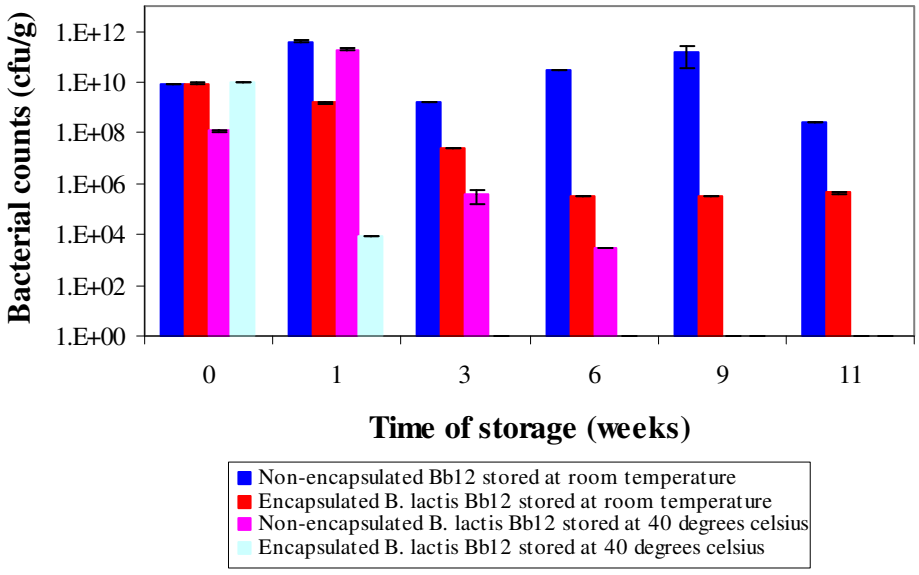

Figure 5.4: Survival of PVP:VA-CA:PEO-PPO-PEO interpolymer complex encapsulated *B. lactis* Bb-12 during storage

Numbers of non-encapsulated bacteria were higher than viable bacteria released from both interpolymer complexes under both storage conditions. The non-encapsulated bacteria counts decreased from 8.80 x 10^9 cfu/g to 2.48 x 10^8 cfu/g at the end of the 11 weeks of storage at room temperature (Fig. 5.4). Counts of viable bifidobacteria released from the complex were lower than the non-encapsulated bacteria counts decreasing from an initial count of 8.87 x 10^9 cfu/g to 4.43 x 10^5 cfu/g at the end of 11 weeks (Fig. 5.4). Encapsulated bacteria had a shelf life of 3 weeks at room temperature, indicated by counts above 1 x 10^6 cfu/g while the non-encapsulated bacteria had a shelf life of more

than 11 weeks (Fig. 5.4). At 40 ^{o}C the reduction in numbers of viable bacteria was faster for both encapsulated and non-encapsulated bacteria. Non-encapsulated counts decreased to 3.63 x 10^{5} cfu/g after 3 weeks of storage while encapsulated bacteria decreased to 8.37 x 10^{3} cfu/g within a week and were undetectable for the rest of analysis period (Fig. 5.4). Acceptable shelf life for non-encapsulated at 40 ^{o}C was 2 weeks. Survival of *B. lactis* Bb-12 was once again better at room temperature than at 40 ^{o}C. Addition of PEO-PPO-PEO triblock copolymer to the interpolymer complex did not improve protection efficiency of the PVP:VA-CA interpolymer complex for bifidobacteria for a 2 h release period before plating. It is possible that if the experiments were repeated for a 6 h release period before plating, that the results may be different.

Effects of PEO-PPO-PEO triblock copolymer addition to PCL:VA-CA interpolymer complex matrix are illustrated in Fig 5.5. Initial viable counts for non-encapsulated bacteria were higher than for the encapsulated. At room temperature, the non-encapsulated bacteria decreased from an initial count of 2.70 x 10^{11} cfu/g to 3.3 x 10^{7} cfu/g at the end of 9 weeks (Fig. 5.5). Levels of the non-encapsulated bacteria remained above the recommended minimum for health benefits throughout the experimental period. Counts of encapsulated bacteria on the other hand decreased from 1.33 x 10^{9} cfu/g to 2.94 x 10^{3} cfu/g at the end of 9 weeks (Fig. 5.5). Acceptable levels of viable bacteria for encapsulated stored at room temperature were retained for a week only.

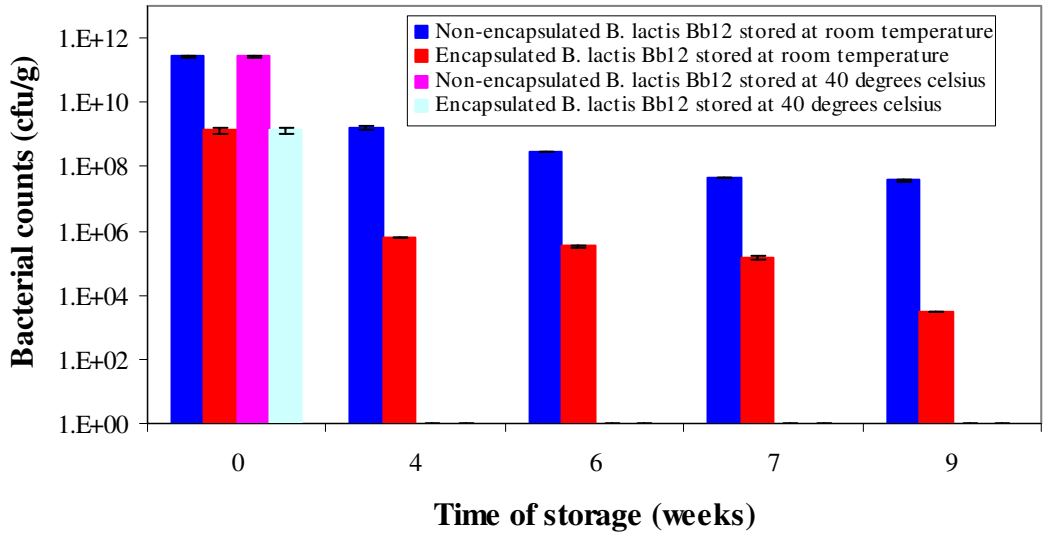

Figure 5.5. Survival of PCL:VA-CA:PEO-PPO-PEO interpolymer complex encapsulated *B. lactis* Bb-12 during storage

There were no viable bacteria detected from both samples after storage at 40 ^{o}C for 4 weeks. It is not clear from the results whether death of cells was faster for non-encapsulated or encapsulated bacteria as analysis was done after 4 weeks. Analysis of samples weekly may have given a better indication of this. Comparing week 9 results it was observed that reduction in numbers of viable bacteria released from the PEO-PPO-PEO:PCL:VA-CA interpolymer complex (Fig. 5.5) was faster than that from PEO-PPO-PEO:PVP:VA-CA matrix (Fig. 5.4). This indicated once again that the complex formed between PVP and VA-CA was stronger leading to better protection than that given by PCL. Nevertheless, incorporation of a flow modifier did not improve shelf life of bacteria in both cases.

5.5 CONCLUSIONS

The PVP:VA-CA interpolymer complex formed in $scCO_2$ provided good protection to *B. lactis* Bb-12 when the encapsulated product was recovered from the reactor and gave negative results when the product was recovered from the product chamber after spraying through a relatively long tube with a heated nozzle at the end. The high shear process

with heating seems to damage the bacteria. In cases where encapsulation provided protection, survival of bacteria was better at lower than at higher temperatures, and/or high relative humidity. The use of polycaprolactone and PEO-PPO-PEO did not improve survival of encapsulated bacteria. Encapsulation seemed to have negative effects on the encapsulated bacteria. This could not be accepted as a reason for low counts as earlier results whereby bacteria were exposed to the encapsulation process indicated that the process did not have any negative effects on the bacterial cells. Not all the encapsulated cells were released from the interpolymer complex for plating out. The 2 h incubation period was not sufficient time for complete release of bacteria and has therefore led to an underestimation of viable bacteria from the matrix as a result of their retention within the interpolymer complex. Since *B. lactis* Bb-12 was found to be one of the strains intrinsically stable to unfavourable conditions, effects of encapsulation could be better demonstrated when more sensitive strains are tested and sufficient time is allowed for complete release of bacteria from the interpolymer complex matrix.

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CHAPTER 6

Shelf life studies of B*ifidobacterium longum* Bb-46 encapsulated in interpolymer complexes in supercritical CO_2 stored under different storage conditions

6.1 ABSTRACT

The effect of interpolymer complex encapsulation on shelf life of *B. longum* Bb-46 was investigated. Encapsulated bacteria were suspended either in sterile ¼ strength Ringer's solution or simulated intestinal fluid and incubated at 30 ^{o}C for 6 h to allow for release of bacteria from the encapsulating matrix. Non-encapsulated bacteria were suspended in Ringer's solution and incubated for 2 h. Serial dilutions of the samples were prepared in sterile ¼ strength Ringers' solution. 0.1 ml of appropriate dilutions was plated out in triplicate onto MRS agar plates supplemented with 0.05 % cysteine hydrochloride. The plates were incubated in anaerobic jars with Anaerocult A gaspaks at 37 ^{o}C for 72 h. Survival in PVP:VA-CA was better when the product was processed at 15 bar product chamber pressure than at 0 bar due to improved particle formation at higher pressure. Incorporation of GMS and beeswax did not give improved protection. However, results indicated that improved release of encapsulated bacteria was obtained when the encapsulated material was incubated in SIF compared to Ringer's solution. When SIF was used, viable counts of encapsulated bacteria remained high up to 12 weeks while levels of non-encapsulated bacteria decreased below the minimum required for beneficial effects after 6 weeks and were undetectable after 10 weeks. Reduction in viable counts was faster for non-encapsulated bacteria than for encapsulated bacteria. Encapsulation in an interpolymer complex therefore provided protection for encapsulated cells and thus has potential for improving shelf life of probiotic cultures in products.

Keywords: encapsulation, simulated intestinal fluid, *Bifidobacterium longum* Bb-46, glyceryl monostearate

6.2 INTRODUCTION

The shelf life of probiotics should be such that products with adequate live bacteria to provide health benefits are manufactured (Kourkoutas et al., 2005). The need for probiotic cultures to remain viable in products during storage can therefore not be overemphasized.

Bifidobacteria differ in their nutrient requirements, growth characteristics and metabolic activity. Thus not all bifidobacteria species will exhibit the same stability in products (Boylston et al., 2004). Different strains from the same species have different beneficial benefits and probiotic properties are also strain specific (Theunissen et al., 2005). Since the properties of bifidobacteria are strain rather than species specific, it will therefore be worthwhile to investigate the effect of microencapsulation on several strains with different properties to find out whether the effect of encapsulation brought upon by microencapsulation on one strain will be the same or different on another strain.

Lian et al. (2002) and Lian et al. (2003) observed that survival of bifidobacteria after spray-drying varied with the strains of test organisms. Hansen et al. (2002) observed that during refrigerated storage in milk different survival improved survival for *B. longum* Bb-46 and *B. lactis* Bb-12 encapsulated in ca-alginate in milk during refrigerated storage. Encapsulation in spray dried whey protein microcapsules improved survival of *B. breve* R070 but not that of *B. longum* R023 during refrigerated storage n yoghurt (Picot and Lacroix, 2004). Boylston et al. (2005) demonstrated different viabilities of different species of bifidobacteria. They found that during refrigerated storage for 14 days, there was a negligible loss in viable counts of *B. bifidum* while there was a reduction of about 3 log cfu/g for *B. infantis* and *B. breve*.

One of the factors that affect shelf life of products is water activity (a_w) (Fontana, 2000). a_w is the most useful expression of water requirements for or water relation of microbial growth and enzyme activity (Troller and Christian, 1978). a_w of a food describes the energy state of water in the food, and hence its potential to act as solvent and participate

in chemical or biochemical reactions and growth of microorganisms. It is used to predict the stability and safety of food with respect to microbial growth, rates of deteriorative reactions and chemical/physical properties (Fontana, 2000). It affects the growth, physiology and metabolism of microorganisms and their resistance to detrimental agents (Liu et al., 1998). It is affected by temperature with the effect being product specific. Different products can either increase or decrease a_w with increasing temperature (Fontana, 2000). It is therefore important that a_w of products during storage is monitored.

Molecular techniques have improved the detection and identification of microorganisms in food microbiology (Theunissen et al., 2005). The potential for application of LIVE/DEAD ® Baclight™ viability kit for rapid and reliable estimation of viable probiotic bacteria was indicated by various researchers (Auty et al., 2001; Moreno et al., 2006). The objective of this study was to investigate the shelf life of *Bifidobacterium longum* Bb-46 encapsulated in interpolymer complexes using plate counts and confocal scanning laser electron microscopy.

6.3 MATERIALS AND METHODS

6.3.1 Encapsulation of bacteria

Bifidobacterrium longum Bb-46 cells were encapsulated in different interpolymer complexes using the method as described in Chapter 5.

6.3.2 Pressing of tablets

The non-encapsulated bacteria and the encapsulated product were pressed into tablets using a Mannesty F3 tablet presser set at 35 cams.

6.3.3 Storage of samples

Both non-encapsulated and encapsulated bacteria samples were stored throughout the experimental period at 30 ^{o}C in sterile glass bottles wrapped in aluminium foil. 1 g samples were taken from the bottles on analysis dates for microscopy and/or plate counts.

6.3.4 Enumeration of bacteria

Two test tubes each containing 9 ml of sterile ¼ strength Ringer's solution (pH 6.8) or simulated intestinal fluid (SIF) (pH 6.8) were prepared. 1 g of encapsulated and non-encapsulated bacteria were added to separate tubes and the tubes were incubated anaerobically at 37 ^{o}C for either 2 or 6 h to allow release of encapsulated bacteria from the interpolymer complex matrix. Serial dilutions of the suspension were made in sterile ¼ strength Ringer's solution. 0.1 ml of appropriate dilutions was pour plated in triplicate onto MRS agar supplemented with 0.05 % cysteine hydrochloride. The plates were incubated anaerobically in anaerobic jars with Anaerocult A gaspaks (Merck), at 37 ^{o}C for 72 h. Anaerocult C test strips were used for indication of anaerobic conditions inside the jars. The numbers of colonies grown were counted and from these the numbers of viable cells were calculated (cfu/g).

6.3.5 Staining of bacteria

The bacteria were stained using double staining at a final concentration of 3.34 µM SYTO 9 nucleic acid stain and 2 µM propidium iodide (PI) in PBS buffer solution. 200 µM of the SYTO 9 and PI mixture was added to the matrix containing bacteria. The stained preparation was incubated in a dark cupboard for 5 min before viewing.

6.3.6 Confocal Laser Scanning Microscopy (CLSM)

Samples were viewed with a PCM2000 Confocal microscope equipped with Argon ion and Helium-Neon lasers. The samples were viewed through a Nikon TE300 inverted microscope using a 60x Plan Apo 1.4 numerical aperture (NA) oil objective. The SYTO 9 stained cells were viewed using Argon Ion laser (480/500 nm) while the PI stained cells were viewed using Helium-Neon Laser (490/635 nm). Images were viewed and saved with the EZ2000 Software.

6.3.7 Water activity (a_w) measurement

The Pa_wKit hand-held water activity meter was used to establish the a_w of the samples. The water activity of the samples was measured following the manufacturer's instructions. Analysis was done in duplicate and the average was reported.

6.4 RESULTS AND DISCUSSION

6.4.1 Shelf life of *B. longum* Bb-46 encapsulated in PVP: VA-CA

The encapsulated bacteria were harvested from the product chamber and incubated in Ringer's solution (pH 6.8) for 2 h for release of encapsulated bacteria, unless specified otherwise. Throughout the storage time of 5 weeks at 30 ^{o}C, viable counts of non-encapsulated bacteria remained above the recommended effective minimum for probiotics. On the other hand, counts of viable bacteria released from the interpolymer matrix processed at 0 bar expansion pressure in the product chamber, were at the levels above 10^8 cfu/g after 2 weeks of storage but were undetectable from 3 weeks onwards (Fig. 6.1). These results indicated the possibility of death of encapsulated cells or alternatively insufficient release of the cells from the interpolymer matrix due to the release media used.

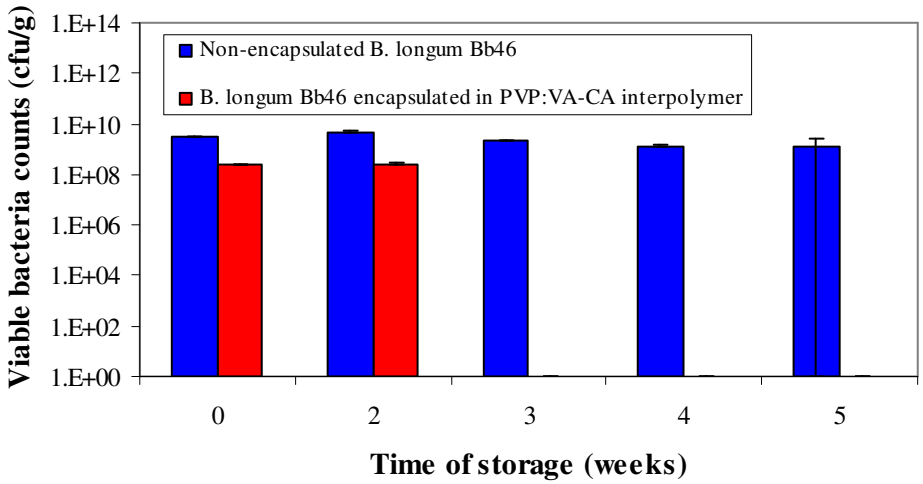

Figure 6.1: Survival of PVP:VA-CA encapsulated *B. longum* Bb-46 processed at 0 bar expansion pressure in the product chamber

The interpolymer matrix may have become harder during storage causing more difficulties in the swelling of the matrix to release the encapsulated cells. This is supported by the high survival of the bacteria when not encapsulated. The absence of viable cells cannot be attributed to the effects of the encapsulating process as earlier work indicated that the process does not affect the bacteria negatively.

Better results were obtained when the product chamber expansion pressure was changed to 15 bar. Even though the non-encapsulated bacteria had higher viable counts than the encapsulated counterparts, the encapsulated bacteria survived better when the pressure was at this level or release of the viable bacteria was not as difficult. The non-encapsulated bacteria remained at levels higher than 10^8 cfu/g throughout the 5 weeks while viable counts of encapsulated bacteria decreased from 10^8 cfu/g to 10^6 cfu/g at the end of the storage period (Fig. 6.2).

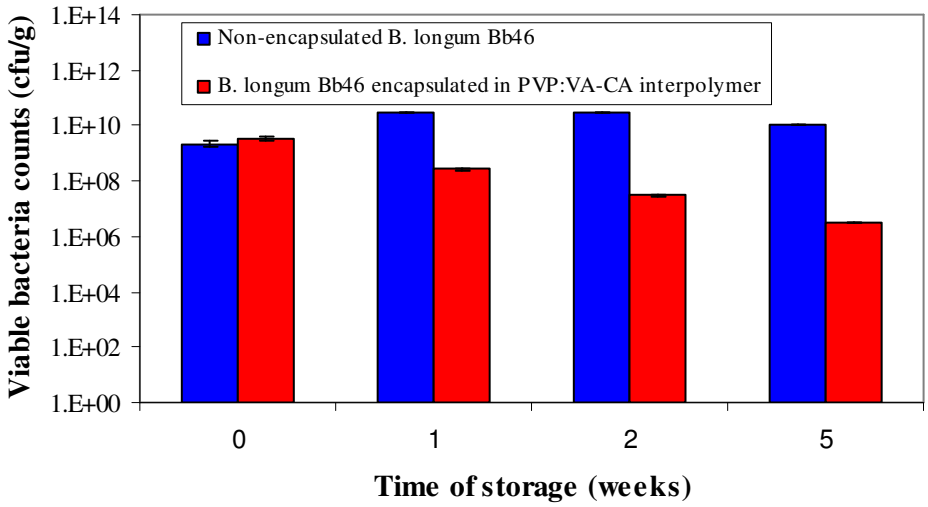

Figure 6.2: Survival of PVP:VA-VA encapsulated *B. longum* Bb-46 processed at 15 bar expansion pressure in the product chamber

An improved result at 15 bar expansion pressure was due to better particle formation than at 0 bar. At 0 bar the material solidified too fast leading to formation of spaghetti-like product which needed extensive grinding before a fine powder could be obtained. On the contrary, at 15 bar a product with short fluffy particles that required less grinding, and hence less chance for bacterial damage, was obtained. Higher pressures (> 30 bar) resulted in slower solidification and splattering of the product on the product chamber walls.

6.4.2 Effect of GMS incorporation on shelf life of *B. longum* Bb-46

Glyceryl monostearate was incorporated in the encapsulation matrix in order to harness its good moisture and oxygen barrier properties to prolong survival of encapsulated probiotics during storage. When GMS was incorporated into the PVP:VA-CA interpolymer complex the results were not changed. Numbers of viable bacteria from the encapsulation matrix were higher than non-encapsulated bacteria during the first 2 weeks only (Fig. 6.3).

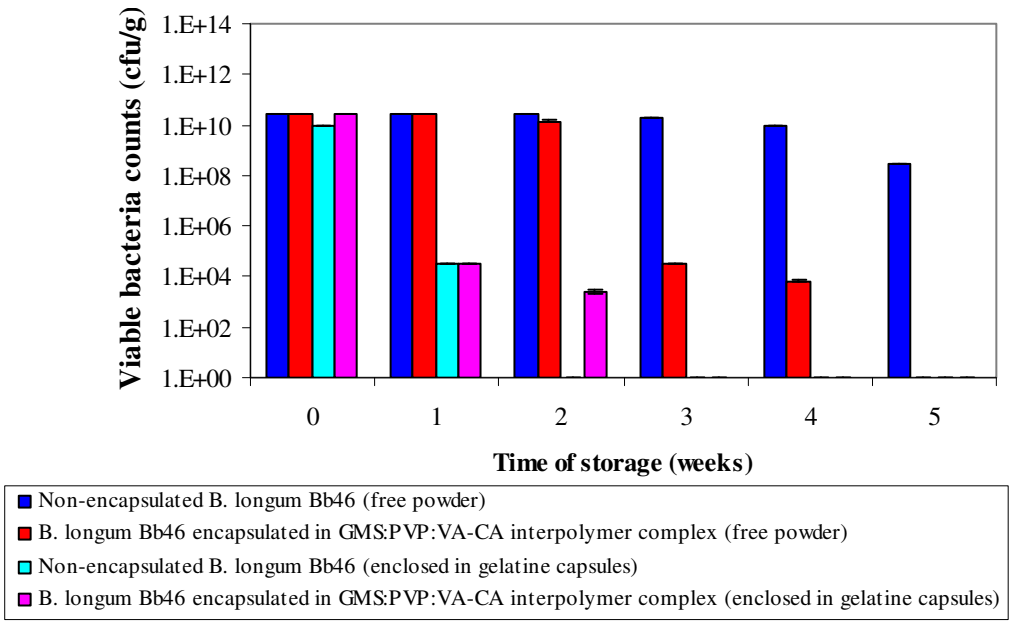

Figure 6.3: Survival of GMS:PVP:VA-CA encapsulated *B. longum* Bb-46 during storage in free powder form and when enclosed into gelatine capsules

After 3 weeks the encapsulated bacteria decreased sharply to levels 50% lower than non-encapsulated bacterial counts. Counts of non-encapsulated bacteria were about 10^8 cfu/g after 5 weeks while no bacteria were counted from the encapsulated material after the same period (Fig. 6.3). Non-encapsulated bacteria counts dropped by about 1 log over the storage period of 5 weeks while the encapsulated bacteria dropped by 5 log cfu/g (Fig. 6.3). Numbers of viable bacteria from GMS incorporated encapsulation formulation were below South African legislation recommended levels of 10^8 cfu/g (Fasoli et al., 2003) at the end of 3 weeks compared to 5 and 9 weeks for non-encapsulated bacteria and encapsulation without GMS (earlier results), respectively. GMS effectively protected the encapsulated bacteria in the early days of storage only. Though higher loadings of GMS to levels as high as 60 % increased survival of encapsulated bacteria in the simulated gastrointestinal fluids (Chapter 4), a similar effect was not observed for shelf life as was anticipated (Data not shown). This was probably also due to use of Ringer's solution and a short incubation time for release of bacteria from the interpolymer complex.

6.4.3 Combined effect of GMS incorporation with either enclosure within gelatine capsules or compression into tablets on shelf life of *B. longum* Bb-46

Viable counts of non-encapsulated bacteria enclosed in gelatine capsules dropped to levels lower than the recommended minimum after only 1 week of storage though they remained at much higher levels when stored in powder form (Fig. 6.3). Viable counts were equal for encapsulated and non-encapsulated bacteria after 1 week of storage (Fig. 6.3). Interestingly, viable bacteria were released from the interpolymer complex even after 2 weeks of storage while the opposite was observed for non-encapsulated bacteria enclosed in capsules. That is, no growth was observed for non-encapsulated bacteria after this period (Fig. 6.3). Absence of viable cells for non-encapsulated bacteria highlighted the negative effects of gelatine capsules on *B. longum* Bb-46 shelf life. This could probably be due to interaction between gelatine capsules and environmental factors such as moisture. Encapsulated bacteria in capsules however lasted for a week more than non-encapsulated bacteria (Fig. 6.3) indicating that the interpolymer complex provided some protection thereby minimizing the detrimental effects to *B. longum* Bb-46 cells brought about by absorption of moisture by capsules. Both the non-encapsulated and encapsulated bacteria survived better when stored in free powder form than when the powders were enclosed in capsules (Fig. 6.3). Enclosure of bacteria in gelatine capsule thus delay the time of exposure of probiotic bacteria to acidic gastric juice (Chapter 4) but has an unfavourable effect on their shelf life. Gelatine capsules are therefore not suitable for packaging probiotics. The main reason for the poor shelf-life of probiotic powders in gelatine capsules is probably the hygroscopicity of the capsule, leading to increased water activity, which in turn would lead to 'activation' of the bacteria from their dormant state (Kell et al., 1998; Lahtinen et al., 2005) and ultimately death.

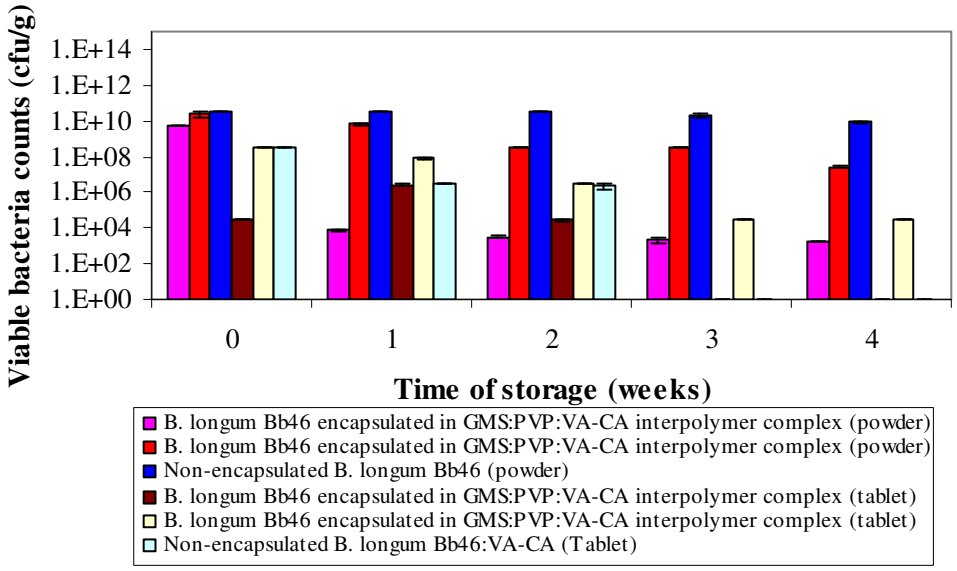

Figure 6.4 Shelf life of *B. longum* Bb-46 encapsulated in presence of GMS during storage at 30 ^{o}C in powder or tablet form

Another form of dosage that could be used for administration of probiotics is tablets. Survival of encapsulated bacteria when pressed into tablets was tested. Tablets were incubated in SIF (pH 6.8) for 2 h while powders were incubated in Ringer's solution for the same period. Survival of bacteria was better when in powder than in tablet form. Viable counts of non-encapsulated bacteria in powder form remained above the recommended minimum throughout the 4 weeks while when in tablet form no viable cells could be detected after 3 weeks of storage (Fig. 6.4). Survival of encapsulated bacteria from separate batches differed. In some instances levels of viable bacteria for encapsulated bacteria in powder form dropped to lower than recommended minimum levels after just a week of storage while in others higher levels were sustained for up to 4 weeks (Fig. 6.4). Viable counts of encapsulated bacteria in tablet form dropped to unacceptable levels after 2 or 3 weeks for different batches (Fig. 6.4). These results indicated the possibility that the process of compressing tablets was detrimental to bacteria even when encapsulated in the interpolymer complex. Also, this process may have hardened the interpolymer complex making it difficult to swell and release the encapsulated bacteria within the 2 h. However, it was worth noting that when both the

non-encapsulated and encapsulated bacteria were stored in tablet form, the encapsulated bacteria survived better than the non-encapsulated bacteria (Fig. 6.4)

6.4.4 Effect of beeswax compared to GMS

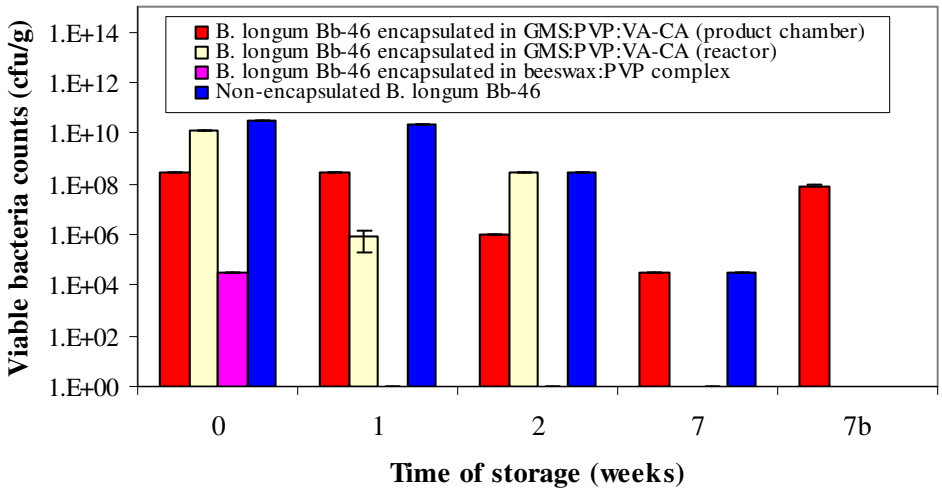

Figure 6.5: Survival of *B. longum* Bb-46 encapsulated in GMS:PVP:VA-CA and beeswax:PVP interpolymer complexes during storage at 30 ^{o}C

Release of encapsulated bacteria from the interpolymer complexes in this case was done in Ringer's solution for 6 h except after 7 weeks of storage when another sample of GMS:PVP:VA-CA encapsulated bacteria from the product chamber was incubated in SIF for 6 h (7b in Fig. 6.5). Counts of all the samples decreased during storage. Viable counts of non-encapsulated bacteria were higher than bacteria encapsulated using both interpolymer matrices for the first 2 weeks, however after 7 weeks their numbers dropped to a level similar to that of viable bacteria released from GMS:PVP:VA-CA interpolymer complex (Fig. 6.5). More viable cells were released from the material recovered from the reactor than from the product chamber. Thus, bacteria from the reactor survived better than those from the product chamber and this was the same observation made when *Bifidobacterium lactis* Bb-12 was the test species (Chapter 5). Their levels after 2 and 7 weeks were equal to non-encapsulated bacteria levels (Fig. 6.5).

The difference in viable numbers between the sample from the reactor and that from the product chamber may in addition to absence of spraying of the product from the reactor as explained in Chapter 5, be as a result of the set-up of the encapsulating system. In the system used, the product chamber is not situated directly under the reactor as is the case with most systems. There is a pipe that extends from the reactor to the product chamber and this may have an influence on the stability of the bacteria. The conditions in the tube that extends from the reactor to the product chamber may negatively affect the bacteria rendering them more sensitive during storage.

The sample from the reactor was just enough for analysis up to after 2 weeks of storage and therefore there were no results for comparison up to 7 weeks. It is therefore not clear whether the stability of this sample was short-lived or whether it survived better than the product chamber sample throughout the storage period.

The plate count results after 7 weeks of storage did not correlate with those of CLSM as images of the latter showed presence of dead cells only (Fig. 6.6, E; F). There were polymer particles on the images which may represent those that were not disintegrated and therefore may still contain live cells (Fig. 6.6, E). The amount of stain used was not enough to release and stain all the cells from the sample.

Presence of viable bacteria within the interpolymer complex stored at 30 ^{o}C for 7 weeks was confirmed by suspension of the sample in SIF instead of Ringer's solution. When the encapsulated product was incubated in SIF at 37 ^{o}C for release of encapsulated bacteria the results indicated that there were still viable cells enclosed within the interpolymer complex, at levels higher than the recommended minimum (Fig. 6.5, indicated on x-axis as week 7b). **These results indicated that proper and sufficient release of encapsulated bacteria was not obtained when Ringer's solution was used and that SIF must be used for suspension and release.**

Beeswax is a foodgrade "polymer" with about 20 % free acid groups which could form a complex with the basic groups of PVP. Beeswax also has very good acid resistance. Incorporation of beeswax slightly improved survival of bacteria in acidic gastric fluid (Chapter 4). The initial viable counts from the beeswax:PVP encapsulated bacteria were lower than 10^6 cfu/g and could not be detected already after 1 week of storage (Fig. 6.5). However, beeswax is not soluble in Ringer's solution, but is digestible in SIF. Thus the counts for the PVP:beeswax system should also have been done using SIF.

CLSM images indicated the presence of live, dead and membrane compromised bacteria after 2 weeks of storage (Fig. 6.6, A; B). The LIVE/DEAD *Baclight* bacterial viability kit was used by other researchers for determining the viability of probiotic bifidobacteria in products (Auty et al., 2001; Lahtinen et al., 2005; Moreno et al., 2006) and for monitoring lysis of cheese starter cultures (Bunthof et al., 2001).

CLSM images of samples stored at 30 ^{o}C for 2 weeks showed the presence of both green and red bacteria, representing live and dead cells, respectively, according to the LIVE/DEAD viability kit manufacturer's instructions. Since SYTO 9 penetrates all the cells and colour them green, the red cells represented dead cells which are permeable to propidium iodide (PI) as well, whose bound SYTO 9 stain was displaced by PI due to its high affinity for DNA (Bunthof et al., 2001), leaving the cells red. There were areas which contained more live cells (Fig. 6.6, A) than dead cells and those which contained more dead cells than live cells (Fig. 6.6, C).

Death of cells will be influenced by the position of cells in the interpolymer matrix whereby cells that are closer to the surface will die faster due to rapid exposure of the cells to detrimental factors such as diffusion of oxygen into the matrix. Therefore, it is likely that areas with more dead cells represent those that were situated closer to the outer regions of the interpolymer complex matrix. It is therefore difficult to conclude whether there were more live or dead cells in these samples due to the difference in the different regions of the sample. It is doubtful whether the stains penetrated the interpolymer complex particles sufficiently to effectively stain all the bacteria throughout the particles.

Also, the penetration depth of the CLSM might not be sufficient to elucidate the state of the bacteria throughout the particles. In addition to dead and live cells, there were also some yellow stained cells. These may represent damaged or compromised cells i.e. those cells that are still in the process of dying or cells that are referred to as viable but non-culturable (Nyström, 2001) or active but non-culturable (Kell et al., 1998), indicating that these cells would be excluded by conventional plating techniques. The yellow colour is due to co-staining with green SYTO 9 and red PI. Low numbers or absence of cells on plates indicated poor release of the interpolymer complex in Ringer's solution.

Samples that were stored for a longer period (7 weeks) showed presence of dead cells only. Although there were patches of green observed it was not clear whether these were still live cells or whether it was stained polymers. There were some green and yellow coloured areas underneath/below the dead cells which may represent live cells which are still entrapped in the matrix (Fig. 6.6, D). It is possible that most if not all of these retained cells will be viable as they would be protected from detrimental conditions of the environment. Also, stained cells may possibly be those that were situated close to the surface of the matrix making them more accessible to factors such as oxygen as it diffuses through the matrix. Dead cells appeared clumped together when compared to live cells or mixtures of live and dead cells. The results obtained from CLSM images correlated with those of plate counts where the numbers of viable bacteria were reduced after 2 weeks of storage and completely absent after a long storage period when suspended in Ringer's solution.

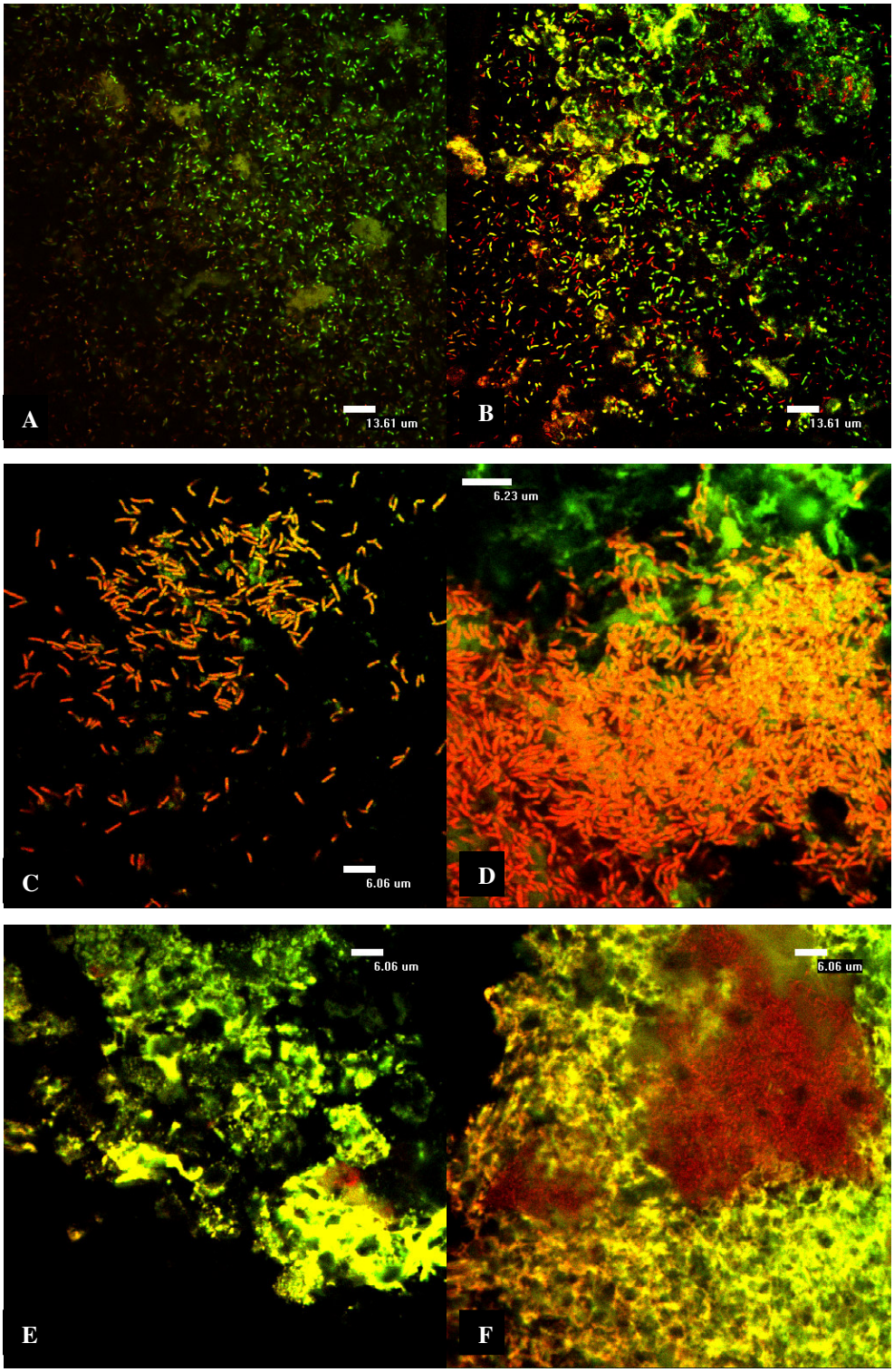

Figure. 6.6:CLSM images of Beeswax:PVP encapsulated *Bifidobacterium longum* Bb-46 stored at 30 ^{o}C for (A; B) 2 weeks, (C; D) 7 weeks and GMS:PVP:VA-CA encapsulated after storage at 30 ^{o}C for 7 weeks (E; F).

6.4.5 Water activities of the samples during storage

Table 6.1: Water activities of samples during storage at 30 ^{o}C as a function of time. The reported value is the average of two readings.

Time (weeks)	Non-encapsulated bacteria	GMS:PVP:VA-CA encapsulated material (product chamber)	GMS:PVP:VA-CA encapsulated material (reactor)	Beeswax:PVP encapsulated material
	a_w			
0	0.25	0.45	0.22	0.53
1	0.28	0.40	0.29	0.55
2	0.41	0.43	0.41	0.60
3	0.39	0.43	0.39	0.56
4	0.50	0.39	0.39	0.58
5	0.40	0.41	0.40	0.59

The a_w values for all the samples were lower at week 0 and higher after 5 weeks of storage at 30 ^{o}C except for GMS:PVP:VA-CA encapsulated material from the product chamber (Table 6.1). The initial a_w of GMS:PVP:VA-CA encapsulated material from the reactor was 0.22 while the material from the product chamber had a a_w of 0.45 (Table 6.1). Since the a_w of the sample from the reactor was lower than of material from the product chamber, these results correlated with the viable counts results which were higher for reactor sample than the product chamber sample as moisture affects stability of bifidobacteria negatively. The a_w of the sample from the reactor was lower than the a_w of the non-encapsulated bacteria which was 0.25 (Table 6.1). The a_w of both these samples was however similar to that of microcapsules produced in the study by Crittenden et al. (2006) for encapsulation of *Bifidobacterium infantis* which was reported to be 0.2-0.3.

These researchers however only reported the initial a_w values of their microcapsules but not readings after any period of storage. Therefore it is unknown whether the a_w of their microcapsules increased, decreased or remained the same during storage. In this study, the a_w of the encapsulated material from the product chamber decreased between week 0 and week 1 and then remained constant from week 2 throughout the storage period while the a_w of the other samples increased. The a_w of beeswax:PVP encapsulated material was the highest, starting at a value of 0.53 and ending at 0.6 at the end of five weeks (Table 6.1). An increase in a_w during storage of this material was similar to that of non-encapsulated bacteria but lower than that of GMS:PVP:VA-CA encapsulated material from the reactor (Table 6.1). The a_w of non-encapsulated bacteria and both GMS:PVP:VA-CA materials were equal at the end of 5 weeks (Table 6.1). However, when looking at the increase in water activity it can be seen that it is too small to solely contribute to death or a reduction in numbers of viable bacteria. From the results, it is very much unlikely that death of bacteria during storage could be an effect of water activity as they indicate that the samples absorb little or no moisture from the surrounding storage environment. Therefore, initial high values for material from the product chamber may have affected the stability of the encapsulated bacteria even though looking at the overall changes during storage, the effect could have not led to the low counts as obtained by plate counts. Water activity values for all the products was however much lower than 0.86 and 0.9 which are the lowest a_w levels at which most spoilage bacteria grow under aerobic and anaerobic conditions, respectively (Fontana, 2000). There were therefore other factors that contributed to lower counts.

6.4.6 Survival of PVP:VA-CA encapsulated bacteria after suspension in SIF for 6 h

Non-encapsulated bacteria started with initial viable counts higher than encapsulated bacteria (Fig. 6.7). Encapsulated bacteria released from the encapsulated product recovered from the reactor were still higher than those from the product from the product chamber (Fig. 6.7). Non-encapsulated bacteria decreased by 6 log cfu/g after 8 weeks of storage at 30 ^{o}C while the encapsulated bacteria decreased by 4 and 3 log cfu/g for product chamber and reactor samples, respectively (Fig. 6.7). No viable counts were

obtained for non-encapsulated bacteria after 10 weeks while viable counts of encapsulated bacteria were 1. 65 x 10^6 cfu/g and 1.25 x 10^7 cfu/g for product chamber and reactor samples, respectively (Fig. 6.7).

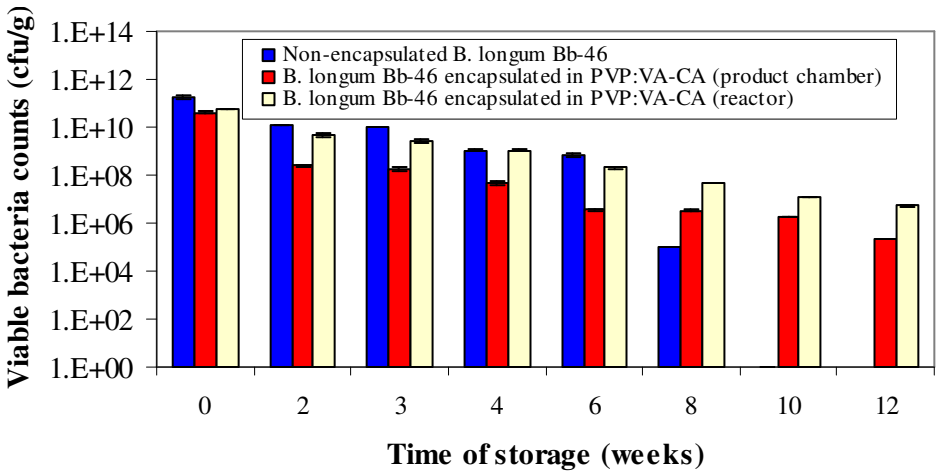

Figure 6.7: Survival of *B. longum* Bb-46 encapsulated in PVP:VA-CA interpolymer complexes after storage at 30 ^oC and release in SIF

Interestingly, even though spraying of the product from the product chamber decreased viable counts when compared to the reactor sample, their levels remained higher than the non-encapsulated bacteria from 8 weeks of storage until the end of the storage period. These results indicate that release of encapsulated bacteria was enhanced in SIF. Non-encapsulated bacteria had acceptable levels of viable bacteria up to 6 weeks of storage while the encapsulated bacteria had sufficient levels for up to 10 and 12 weeks for product chamber and reactor samples, respectively (Fig. 6.7). Thus encapsulation increased shelf life of *B. longum* Bb-46 cells by 4 weeks when the product was recovered from the product chamber and by 6 weeks (indicating that shelf life was doubled) when the product was recovered from the reactor. Therefore encapsulation has potential for improving the shelf life of probiotics in products

6.5 CONCLUSIONS

Encapsulated bacteria survived better at 15 bar product chamber expansion pressure than at 0 bar as a result of better particle formation at 15 bar. Incomplete release of encapsulated bacteria from the interpolymer complex due to use of Ringer's solution resulted in an underestimation of viable counts for encapsulated bacteria when compared to non-encapsulated bacteria. Encapsulated bacteria survive better when stored in powder form than when pressed into tablets or enclosed into gelatine capsules. Gelatine capsules are unsuitable for packaging of probiotics and tablets are not recommended as a delivery form of probiotics. On the other hand, the tablet results were determined using Ringer's solution – the lower counts compared to powder for the encapsulated bacteria might have been due to even less effective release because of the compact tablet and the low solubility/swelling in Ringer's solution of the interpolymer complex. The control results do point towards bacteria damage due to the tabletting process. More stable encapsulated probiotics are obtained when the product is recovered from the reactor compared to the product chamber. Ringer's solution does not give proper and sufficient release of encapsulated bacteria from the matrix. Simulated intestinal fluid on the other hand allows sufficient release of encapsulated bacteria from the matrix and should therefore be used for release. The earlier results obtained can not be properly interpreted due to the use of Ringer's solution instead of SIF, as well as a short release time, which lead to incomplete release from the interpolymer complex. The products absorb little or no moisture during storage, causing a slight change in a_w. Therefore reduction in numbers of viable cells is probably not as a result of a_w. Suspension of encapsulated bacteria in SIF allowed sufficient release of encapsulated bacteria from the interpolymer complex showing better survival of encapsulated bacteria than non-encapsulated bacteria. Encapsulation improved survival of *B. longum* Bb-46 during storage therefore has potential for improving shelf life of probiotics.

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CHAPTER 7

GENERAL CONCLUSIONS

- South African probiotic yoghurts are not adequately labelled with regard to numbers of viable cultures in cfu/ml present in products at the end of their shelf lives, and with regards to proper identification of incorporated microorganisms up to genus and species level.

- There is generally a poor stability of probiotic bifidobacteria in products. Lactobacilli however are more stable in products and generally retain recommended levels for beneficial effects

- Scanning electron microscopy indicates that PVP and VA-CA are liquefiable in supercritical CO_2 allowing successful encapsulation of *B. lactis* Bb-12 and *B. longum* Bb-46 in the interpolymer complex

- The encapsulation process does not produce visible morphological changes to bacteria nor negatively affect viability of bacteria. High numbers of viable probiotic cultures can be encapsulated and then released from the interpolymer complex.

- The interpolymer complex has pH- responsive release properties with little to no release of encapsulated bacteria in low pH of the SGF and substantial release in high pH of the SIF. There are however batch-to-batch variations in the initial counts of encapsulated bacteria indicating different pre-exposures of the freeze-dried bacteria and with regard to release and protection of bacteria by the same interpolymer complex.

- Addition of 8 % GMS improves the protection efficiency of the basic system, PVP:VA-CA. Increasing the concentration of GMS in the interpolymer complex to as high as 60 % enhances the protective effect of the complex further.

- Alternative complexes such as PCL:VA-CA and beeswax:PVP do not protect encapsulated bacteria from the gastric acidity to the same extent as the PVP:VA-

CA system. Addition of PEO-PPO-PEO (a processing agent) to both PVP:VA-CA and PCL:VA-CA interpolymer complexes has undesirable effects, weakening the complexes and thus resulting in release of more of the encapsulated cells in simulated gastric fluid. Shelf life of bacteria encapsulated in these interpolymer complexes is decreased by addition of PEO-PPO-PEO.

- PVP:VA-CA interpolymer complex increases the shelf life of *B. lactis* Bb-12 under all storage conditions when the product is recovered from the reactor compared to when recovered from the product chamber. Encapsulation doubles the shelf life of *B. longum* Bb-46.

- Survival of bacteria is better at lower than at higher temperatures. The humidity chamber is the most unfavourable condition for storage

- The products absorb little or no moisture during storage, causing a slight change in a_w.

- Survival of bacteria is better when the product is stored in glass bottles than when stored in plastic bags. Encapsulated bacteria survive better when stored in powder form than when pressed into tablets or enclosed into gelatine capsules.

- A more representative release of bacteria from the interpolymer complex is obtained when incubation was in simulated intestinal fluid for 6 h compared to Ringer's solution.

- Encapsulation in an interpolymer complex has potential for protecting probiotic cultures from the acidity of the stomach and improving their shelf life in products.

APPENDIX

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Report : Survivability of encapsulated *Bifidobacterium longum* under *in vitro* gastrointestinal conditions

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Ghent, February 17, 2007

Introduction

Probiotics are living microorganisms that, when ingested, beneficially affect the host's health. They are commonly dosed as food supplements under the form of yoghurts or capsules. There are different mechanisms by which probiotic organisms have positive effects towards the host. The group of probiotic lactobacilli for instance produce lactic and acetic acid by which they lower the pH in the intestinal environment, thus creating growth-inhibiting conditions for opportunistic pathogens. Some lactobacilli also produce bacteriocins by which they have a direct inhibitory effect against pathogens and other probiotic organisms go into competition with pathogens for nutrients. Some probiotic strains adhere to the intestinal epithelium by which they prevent the adherence of pathogenic bacteria. Moreover, some probiotics stimulate the host's immune system by increasing the epithelial production of immunoglobulins.

The most important criteria for microorganisms to come into consideration for being applied as a probiotic are survival in the acidic environment of the stomach, resistance against digestive enzymes and bacteriostatic bile salts in the small intestine, survivability in the complex microbial community of the large intestine and ability to decrease the adherence and/or growth of pathogenic bacteria in the gastrointestinal tract.

Bifidobacterium longum is one of the best studied probiotic bacteria from the group of bifidobacteria. The genome has been fully sequenced and its probiotic effects in human have been well-described. For instance, *Bifidobacterium longum* has been successfully applied to abate lactose intolerance, to relieve gastrointestinal complaints from radiation sickness and increase the colonization resistance against pathogens. Up till now, *Bifidobacterium longum* has been typically applied in a dosage range of 9 to 11 log CFU/mL. This way, enough bacterial cells survived the stomach and small intestine passage in order to ensure a high enough concentration reaching the large intestine environment that may then elicit health-promoting effects.

Yet, increasing the survival of this probiotic strain during gastrointestinal passage may still enhance the effectiveness of this probiotic. One of the novel techniques that is under study is encapsulation which would make a strain stable, convenient to administer and amenable to widespread use. In this study, it was evaluated to what extent encapsulation of *Bifidobacerium longum* increased its survival during stomach and small intestine passage and to what extent the added strain was able to maintain itself in the complex microbial community from the large intestine. For this, the Simulator of the Human Intestinal Microbial Ecosystem was used.

Material and methods

Products of *Bifidobacterium longum*

Two products were tested and compared to one another. A first product contained *Bifidobacterium longum* in lyophilized form and is further referred to as Bif L. The second product contained *Bifidobacterium longum* in the encapsulated form and is further referred to as Bif E.

Reactor setup

The Simulator of the Human Intestinal Microbial Ecosystem (SHIME) is a dynamic model of the human gastrointestinal tract. It consists of 5 double-jacketed vessels maintained at a temperature of 37 °C, respectively simulating the stomach, small intestine, ascending colon, transverse colon and descending colon, with a total retention time of 76 h. The colon vessels harbour a mixed microbial community and pH controllers (pH controller R301, Consort, Turnhout, Belgium) maintain the pH in the range 5.6-5.9, 6.2-6.5 and 6.6-6.9 in the ascending, transverse and descending colon simulations, respectively. There is no gas exchange between the different vessels and the headspace of the culture system was flushed twice a day for 15 min with N_2 to ensure anaerobic conditions. The growth medium for the microbial inoculum consisted of a carbohydrate-based medium containing arabinogalactan (1 g l-1), pectin (2 g l-1), xylan (1 g l-1), starch

(4.2 g l-1), glucose (0.4 g l-1), yeast extract (3 g l-1), peptone (1 g l-1), mucin (4 g l-1) and cysteine (0.5 g l-1).

For the survival of both *Bifidobacterium* products, a worst case scenario was envisaged. Here, a stomach pH of 2 was maintained during 2 hours after which the stomach suspension with Bif L or Bif E was gradually transferred to the small intestine compartment where digestion with pancreatic enzymes and bacteriostatic bile salts takes place.

Experimental setup

The SHIME reactor was operated during 12 weeks and consisted of the following periods:

- Stabilization period: week 1, 2 and 3
- Control period: week 4 and 5
- Treatment period Bif L: week 6
- Washout period Bif L: week 7
- Treatment period Bif E: week 8
- Washout period Bif E: week 9
- Prolonged washout and monitoring period: week 10, 11 and 12

At the beginning of the stabilization period, the SHIME colon vessels were inoculated with isolated fecal microorganisms. During the first 3 weeks of the SHIME run, different microbial communities develop in the respective colon vessels, due to changes in parameters such as incoming digested food, pH, redox potential… After three weeks, the microbial communities in the ascending, transverse and descending colon vessels differ from one another in composition and metabolic activity and resemble the microbial community from the respective colon compartments *in vivo.*

The major objective during the control period was monitoring the baseline levels of fermentation activity and determination of the microbial community in the different colon vessels. This way, it could be assessed to what extent the supplementation of Bif L or Bif

E influenced the residing microbial community or Bifidobacteria population in the colon vessels. During the treatment period Bif L, 1 g of Bif L was supplemented daily to the SHIME reactor. It was subjected to the stomach and small intestine digestion steps and was then transferred to the colon compartments. During the washout period Bif L, no supplementation of the *Bifidobacterium* product was carried out. During the treatment period Bif E, 1 go of Bif E was supplemented daily to the SHIME reactor. It was subjected to the stomach and small intestine digestion steps and was then transferred to the colon compartments. During the washout period Bif E, there was no more supplementation of Bif E to the SHIME. The washout period was prolonged to see whether Bif E had an long term effect on the metabolic activity or community composition of the SHIME colon compartments.

Analyses

Short chain fatty acids (SCFA). Liquid samples were collected and frozen at -20 °C for subsequent analysis. The SCFA were extracted from the samples with diethyl ether and determined with a Di200 gas chromatograph (GC; Shimadzu, 's-Hertogenbosch, The Netherlands). The GC was equipped with a capillary free fatty acid packed column (EC-1000 Econo-Cap column (Alltech, Laarne, Belgium), 25 mx0.53 mm; film thickness 1.2 µm), a flame ionization detector and a Delsi Nermag 31 integrator (Thermo Separation Products, Wilrijk, Belgium). Nitrogen was used as carrier gas at a flow rate of 20 mL min-1. The column temperature and the temperature of the injector and detector were set at 130 °C and 195 °C, respectively.

Ammonia. Using a 1026 Kjeltec Auto Distillation (FOSS Benelux, Amersfoort, The Netherlands), ammonium in the sample was liberated as ammonia by the addition of an alkali (MgO). The released ammonia was distilled from the sample into a boric acid solution. The solution was back-titrated using a 665 Dosimat (Metrohm, Berchem, Belgium) and 686 Titroprocessor (Metrohm).

Microbial community analysis.

Plate counting. The following bacterial groups were quantified by growth on specific media (Oxoid, Hampshire, UK): lactobacilli (rogosa agar), bifidobacteria (raffinose *Bifidobacterium* agar), enterococci (*Enterococcus* agar), enterobacteria (MacConkey agar), and clostridia (tryptose sulfite cycloserin agar). Liquid samples were withdrawn from the culture system and serially diluted in saline solution (8.5 g NaCl 1^{-1}). Three plates were inoculated with 0.1 ml sample of three dilutions, and incubated at 37 °C (43 °C for enterobacteria) under aerobic or anaerobic conditions where necessary. Anaerobic incubation of plates was performed in jars with a gas atmosphere (84% N_2, 8% CO_2, and 8% H_2) adjusted by the Anoxomat 8000 system (Mart, Sint-Genesius-Rode, Belgium).

PCR-DGGE. The bifidobacteria population of the colon was analysed. A nested PCR approach was used to amplify the 16S ribosomal RNA genes of the bifidobacteria. In brief, one µl of the DNA was amplified using the primers BIF164f-BIF662r for bifidobacteria. When the first PCR round produced a clearly visible band, a second amplification round with forward primer P338F (with a GC-clamp of 40 bp) and reverse primer P518r was used. The 16S rRNA genes of all bacteria were amplified applying primers P338F with GC-clamp and P518r on total extracted DNA. Denaturing gradient gel electrophoresis was performed as described earlier using the Bio-Rad D Gene System (Bio-Rad, Hercules, CA, USA). PCR fragments were loaded onto 8% (w/v) polyacrylamide gels in 1 × TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 7.4). On each gel, a home made marker of different PCR fragments was loaded, which was required for processing and comparing the different gels (Boon *et al.* 2002). The polyacrylamide gels were made with denaturing gradient ranging from 45% to 60%. The electrophoresis was run for 16 h at 60 °C and 38 V. Staining and analysis of the gels was performed as described previously described.

Real-time PCR. For quantification of bifidobacteria by real-time PCR, amplification was performed in 25 µl reaction mixtures by using buffers supplied with the qPCRe Core

Kit for Sybre Green I as described by the suppliers (Eurogentec, Liège, Belgium) in Micro-Amp Optical 96-well reaction plates with optical caps (PE Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands). Primers BIF164f-BIF662r for 16S ribosomal RNA genes were used for the quantification of bifidobacteria at a concentration of 1 µmol l-1. PCR temperature program was as follows: 50°C for 2 min, 95 °C for 10 min, followed by 40 cycles of 94°C for 1 min, 62°C for 1 min and 60°C for 1 min. The template DNA in the reaction mixtures was amplified (n = 3) and monitored with an ABI Prism SDS 7000 instrument (PE Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands). DNA was extracted from a 6.4×10^7 CFU/ml culture of Bifidobacterium breve (LMG11042). Standard curves were constructed after real-time PCR amplification of four different DNA concentrations (n = 4) ranging from 1.28×10^7 to 1.28 × 104 cell equivalents/well. The standard curve had a R^2 value of 0.99 and the slope was -3.3.

Results and discussion

Specific

It should be mentioned that on during the treatment period Bif E, a white precipitate was formed in the stomach compartment. This white precipitate was transferred to the small intestine compartment and clogged the tubings on day 3 of treatment period Bif E which lead to a technical breakdown of the SHIME. All tubings from the stomach and small intestine vessels were replaced and the SHIME experiment was resumed that same day.

Survival of the strain after stomach and small intestine digestion

To find out to what extent Bif L was able to survive the gastrointestinal passage, samples were taken from the stomach and small intestine compartments from the SHIME after the digestions in those respective compartments had finished. These experiments were repeated 3 times: on day 1, day 4 and day 7 of the treatment period Bif L. The inoculum concentration, as measured by plate counts was 8.4 log CFU/mL (Figure 1). After the stomach digestion a very significant decrease of Bif L to values between 5.5 and 5.8 log

CFU/mL was observed. In the small intestine, a higher survival of Bif L was noted with values between 6.6 and 7.6 log CFU/mL of small intestine suspension (Figure 1).

The same measurements were done during treatment period Bif E. As the product Bif E was delivered at LabMET at the end of the day, plate counts of Bif E in the stomach and small intestine were not made. The survival of Bif E was therefore measured two times: on day 5 and day 7. It was immediately noted that the plate count measurement of Bif E in the inoculum was only 4.7 log CFU/mL. This was probably due to the fact that not all bacterial cells in the product could be measured by plate counting as they were still encapsulated. Nevertheless, a very high survival of this strain was noted in the stomach compartment both on day 5 (4.8 log CFU/mL) as on day 7 (5.4 log CFU/mL).

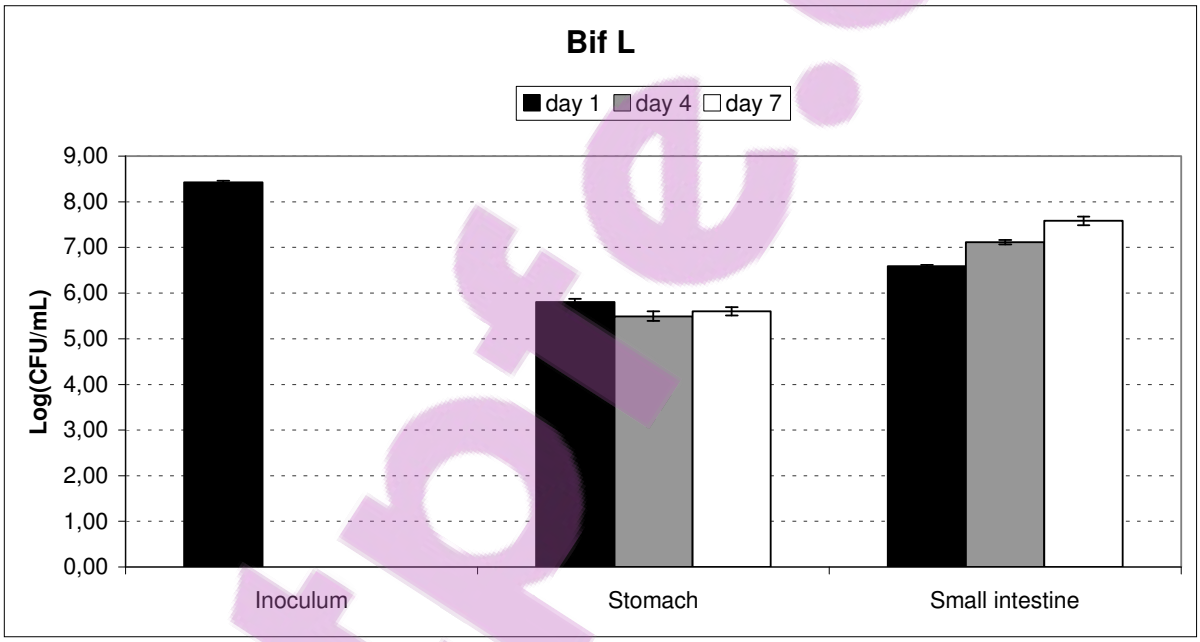


Figure 1. Plate count measurements of *Bifidobacterium longum* in product Bif L after inoculation and after the stomach and small intestine passage on day 1, day 4 and day 7 of treatment period Bif L

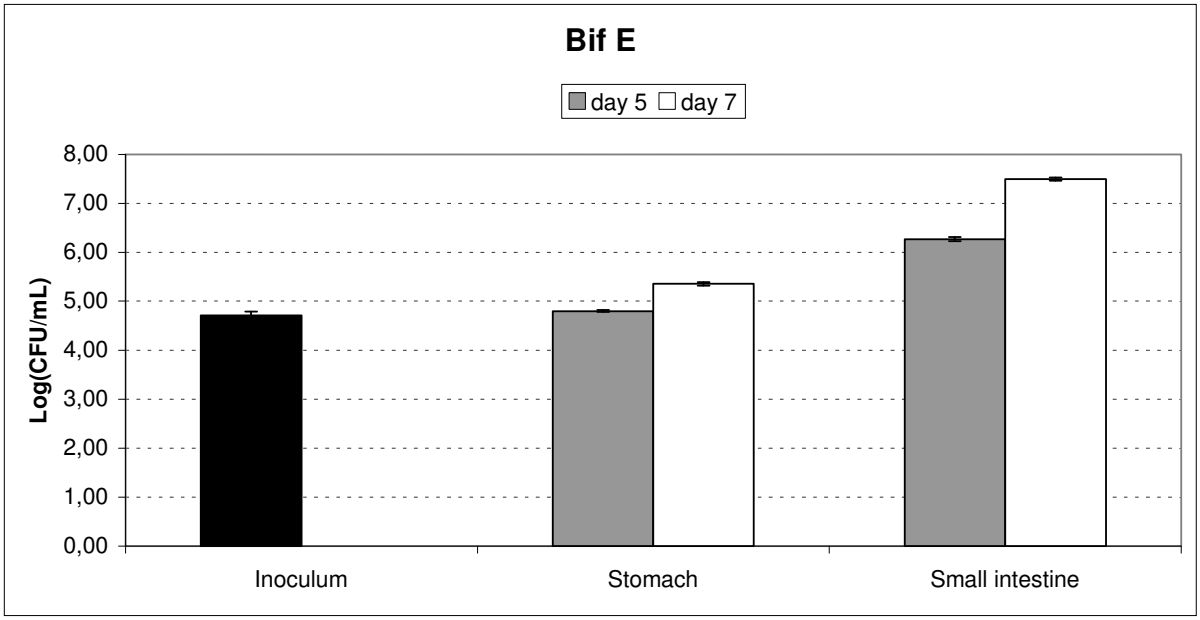


Figure 2. Plate count measurements of *Bifidobacterium longum* in product Bif E after inoculation and after the stomach and small intestine passage on day 1 (inoculum concentration solely), day 5 and day 7 of treatment period Bif E

As with Bif L, a higher concentration of *Bifidobacterium longum* was observed in the small intestine, 6.3 and 7.5 log CFU/mL on day 5 and 7 of treatment period Bif E, respectively. As not all bifidobacteria cells in the suspension may have been counted – due to the encapsulation process – these values may be an underestimation of the actual amount of bifidobacteria in the suspension.

To deal with the fact that not all bifidobacteria may have been counted from the encapsulate product Bif E, real-time PCR was carried out on the samples from the stomach and small intestine. For Bif L, a similar trend as with the plate counts was observed. For Bif L in the stomach sample a concentration of around 5 log copies/mL was noted, whereas in the small intestine, a higher concentration of around 7 log copies/mL was observed (Figure 2). Interestingly, the encapsulated product Bif E showed a very high concentration of bifidobacteria both in the stomach as the small intestine sample (Figure 2). This indicates that the encapsulation process for product Bif E has actually worked very well and that the strain is able to survive the harsh conditions

from the stomach and small intestine. It should be noted that product Bif L eventually also had a substantial survival in the small intestine. This product as well would have a chance of reaching the colon environment in a high concentration. Yet, the survival of the encapsulated *Bifidobacterium longum* surpasses that of the lyophilized strain by several orders of magnitude.

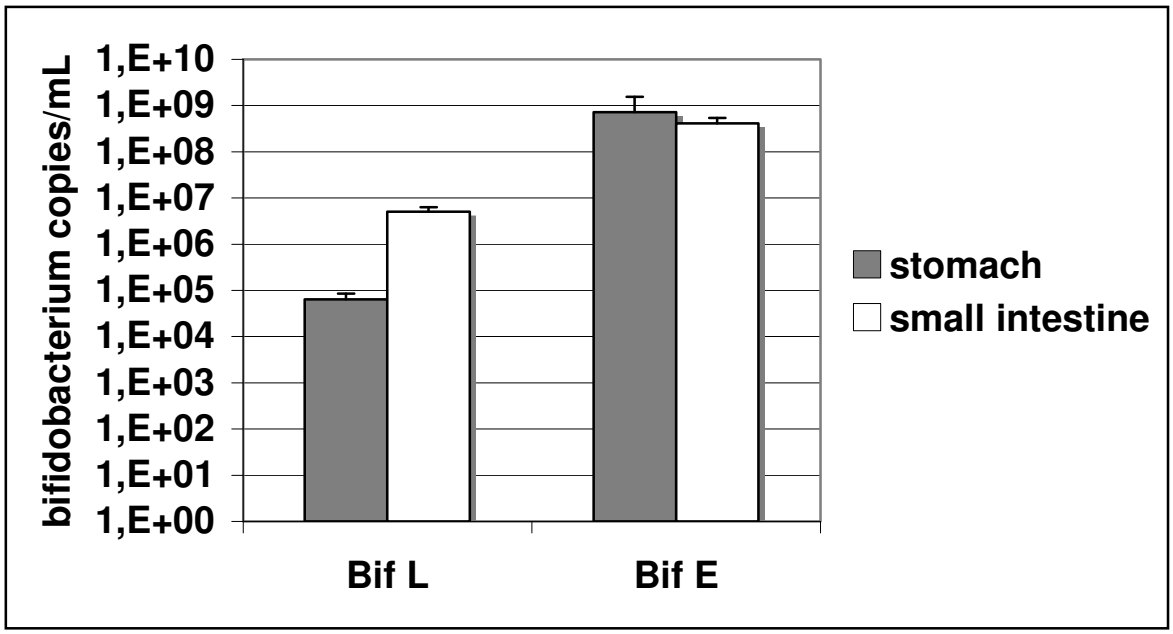


Figure 2. Real-time PCR analysis for bifidobacteria in the samples from the stomach and small intestine during the treatment with product Bif L and product Bif E.

Influence of Bif L and Bif E supplementation on the colon microbial community

Fermentation activity

During the entire SHIME run, no specific differences were observed in short chain fatty acid production (Figure 3). At the end of washout period Bif E, a temporary increasing trend in propionic and acetic acid production was observed (Figure 3A and B). There is however no evidence that this was attributed to the prolonged presence of the encapsulated Bif E product. Similarly, during the entire SHIME run, no specific differences were observed in ammonia production (Figure 4). This indicates that the

supplementation of Bif E or Bif L, if they survived the colon during the SHIME run, did not interfere too much with the basic metabolic activities from the intestinal microbiota.

Microbial community composition

Plate count measurements from the most important microbial groups indicated no significant changes in microbial composition that could be explained by the treatment with Bif L or Bif E (Figure 5). This indicates that the supplementation of Bif E or Bif L did not interfere too much with different microbial groups of relevance in the SHIME colon compartments. No significant increase in bifidobacteria counts was observed during week 6 and 8 (the two weeks of treatment). Probably, the added *Bifidobacterium longum* did not reach the colon compartments in a high enough concentration to be detectable amongst the other bifidobacteria in the colon suspension. Therefore, a molecular strategy was applied to investigate whether the strain was detectable in the colon suspension.

Bifidobacteria DGGE

Using PCR-DGGE, it was attempted to visualize the added *Bifidobacterium longum* in the bifidobacteria population from the different colon compartments. Bacterial DNA was extracted from the intestinal suspension of the colon compartments during the SHIME run and PCR was carried out with specific bifidobacteria primers. Then, a DGGE was made which generates a distinctive fingerprint profile of the bifidobacteria population in the intestinal suspension. Typically, each band in such a DGGE profile roughly corresponds to 1 *Bifidobacterium* species.

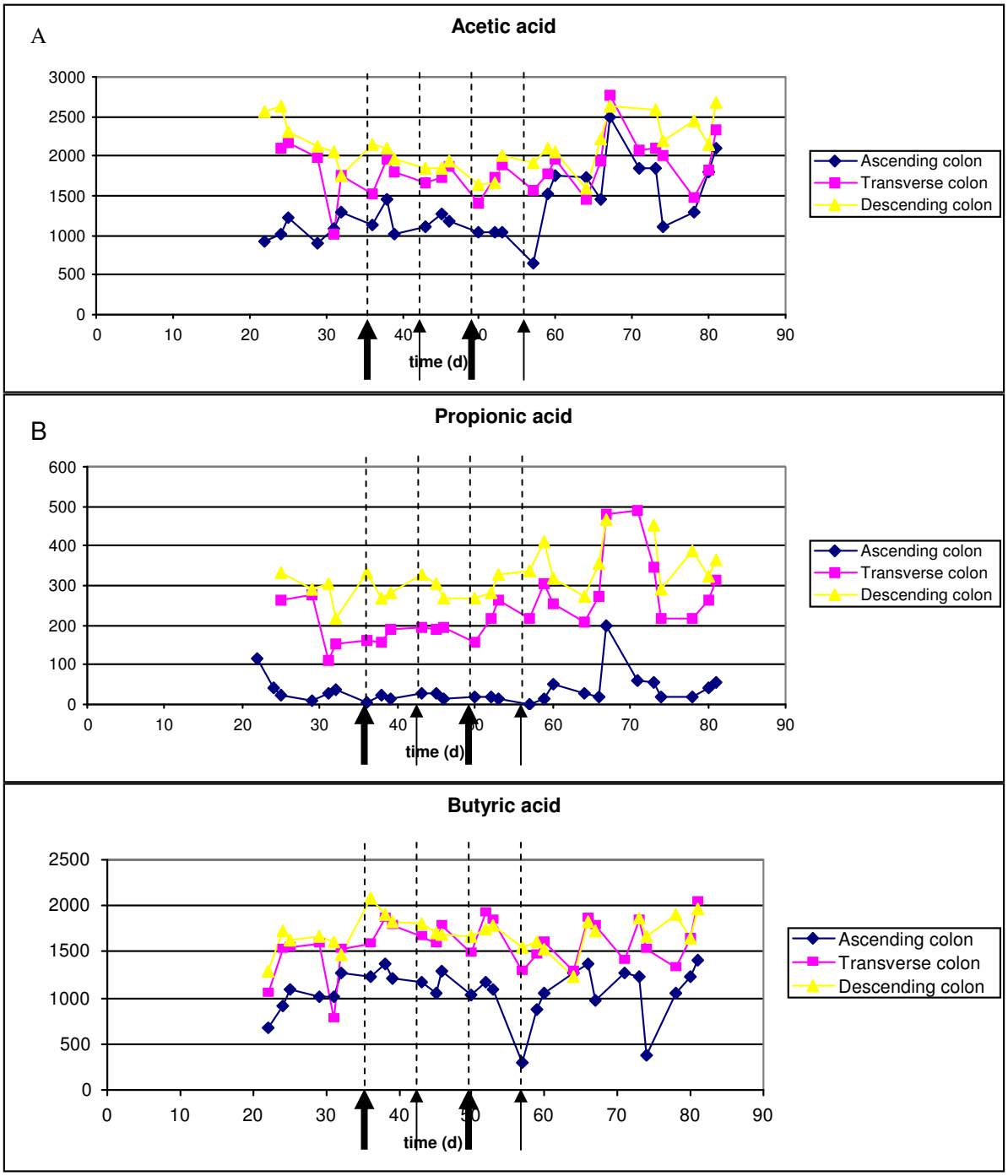


Figure 3. Concentrations of acetic (A), propionic (B) and butyric (C) acid in the ascending, transverse and descending colon vessels of the SHIME reactor. The two bold arrows in each graph indicate the start of the treatment period with Bif L, respectively Bif E. The two normal arrows indicate the start of the respective washout periods.

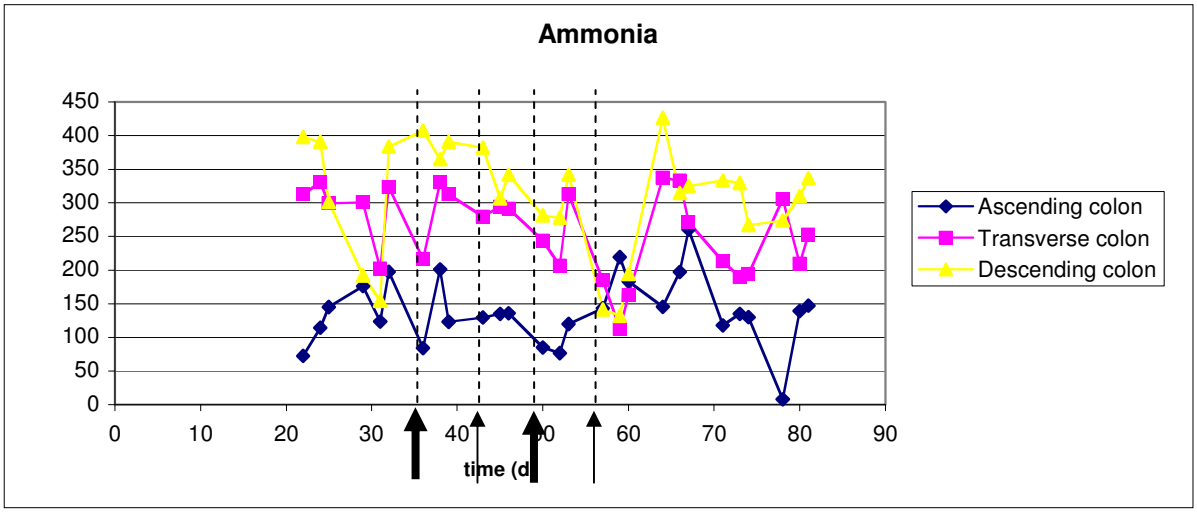


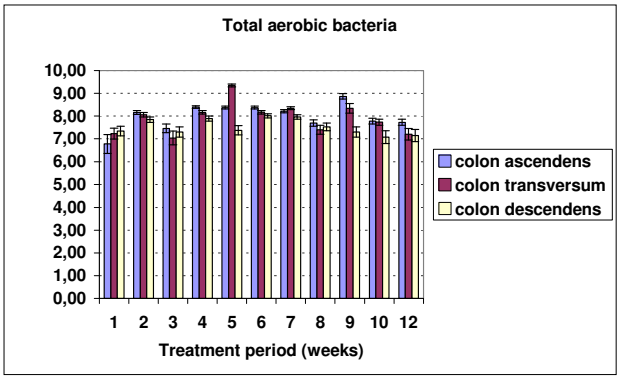
Figure 4. Concentrations of ammonia in the ascending, transverse and descending colon vessels of the SHIME reactor. The two bold arrows in each graph indicate the start of the treatment period with Bif L, respectively Bif E. The two normal arrows indicate the start of the respective washout periods.

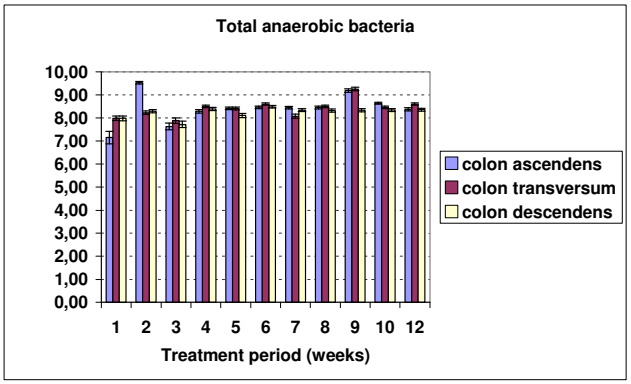
It was noted from the sample of all colon compartments of week 5 (control week, before treatment with Bif L), that quite some bifidobacteria species were visualized on the DGGE (Figure 6). Remarkably, in week 6 (treatment period Bif L) the intensity of one of the DGGE bands was much higher, indicating a higher predominance of that specific *Bifidobacterium* species. The band is marked with a circle in Figure 5. In the following washout period (week 7), the intensity of that specific band got weaker which points to a lower predominance of that species in the washout week. In the following week (week 8), treatment period Bif E, the specific band got more intense again in all 3 colon compartments. The following washout period (sample of week 9) brought again a decrease in the strain.

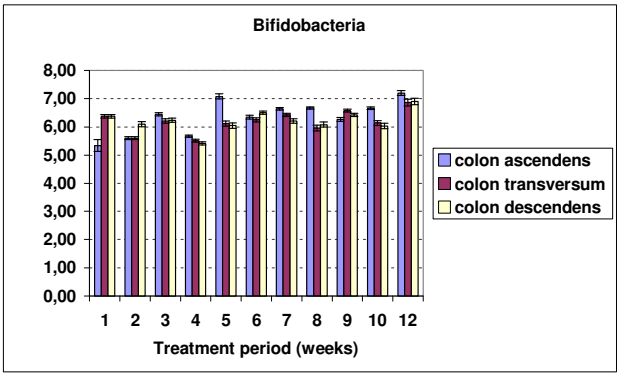
Presumably, the bacterial species that is responsible for the more intense DGGE band, is the added *Bifidobacterium longum* from the Bif L and Bif E products. This was confirmed by another DGGE analysis where *Bifidobacterium longum* from product Bif L was put on gel together with the colon samples from the treatment period (data not shown). Bif L generated a band that appeared on DGGE at the same height as the bands

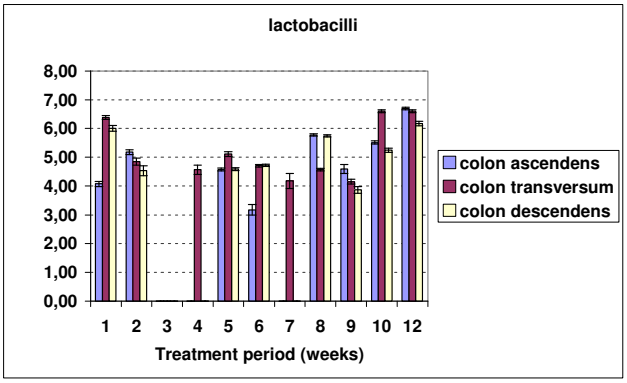
that got more intense in samples from the colon compartments during the treatment periods Bif L and Bif E.

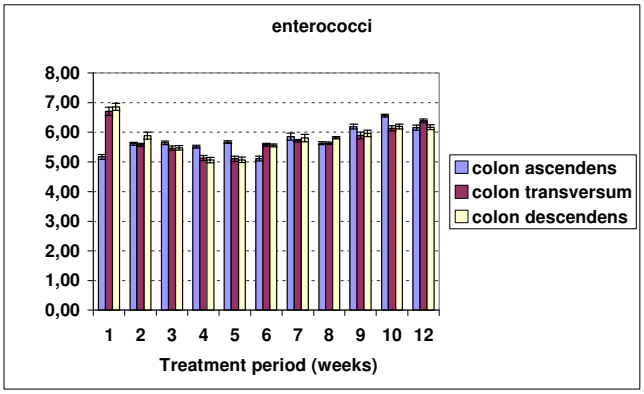
Further research is currently underway to quantify the supplemented *Bifidobacterium longum* by means of real-time PCR. The development of this protocol is however still underway.











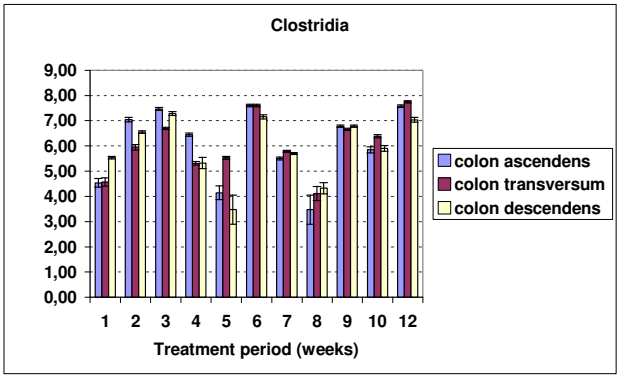


Figure 5. Plate count measurements of the most relevant microbial groups in the ascending, transverse and descending colon vessels of the SHIME reactor. Week 6 and week 8 were the treatment periods with product Bif L and Bif E, respectively.

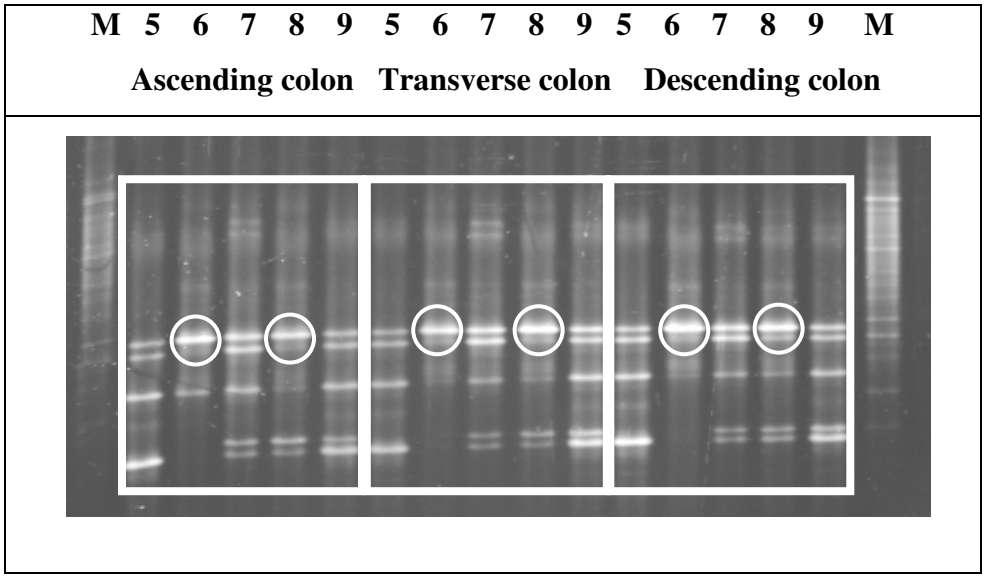


Figure 6. DGGE pattern of bifidobacteria in the colon compartments of the SHIME reactor. "M" indicates marker lanes. Samples from the respective colon compartments are grouped together. Week 6 and 8 were the treatment weeks with product Bif L and Bif E respectively. The band that was more intense in the DGGE profile during the treatment periods is indicated with a white circle.

Conclusion

Two products of *Bifidobacterium longum* were tested in this SHIME experiment: a lyophilized form of this bacterium Bif L and an encapsulated form Bif E. When Bif E was added, a recalcitrant precipitate was formed in the stomach compartment for which caution should be taken in future research steps. Bif L showed a moderate degree of survival during stomach passage and a higher degree of survival during small intestine passage. In contrast Bif E showed a very high survival under stomach conditions as well as under small intestine conditions. Although plate counts showed that not all bifidobacteria were measurable in the samples that contained Bif E (due to the

encapsulation process), real-time PCR data showed strong evidence that survival of Bif E during the stomach passage was almost complete.

With regard to the influence of Bif L and Bif E on the colon microbial community, no interference with the most important metabolic processes in the colon compartments was observed. Although it was expected that the higher survival of Bif E would have resulted in beneficial shifts in the fermentation pattern, no changes in short chain fatty acid or ammonia production were noted. Likewise, no significant changes in microbial community were observed by plate count measurements, nor in the health-promoting population (bifidobacteria and lactobacilli) nor in the more pathogenic population (clostridia).

Interestingly, DGGE profiles of the bifidobacteria population showed that the added probiotic strain was enriched in the population, both during addition of Bif L as Bif E. It should however be remarked that the PCR-DGGE analysis also detects inactive bacteria. It can therefore not be assessed to what extent the added *Bifidobacterium longum* maintained itself in the colon suspension. No prolonged presence of the strain was observed in the periods following the treatment, indicating that the supplemented strain had probably washed out. However, real-time PCR analysis for the *Bifidobacterium longum* strain is currently under development for a better quantification of the specific strain in the colon suspension. This will allow to assess to what extent the supplemented probiotic strain persists in the colon compartments during the treatment, but also during the washout periods.