

**Poly-(vinylpyrrolidone)-poly-(vinylacetate-co-crotonic acid) (PVP: PVAc-CA) interpolymer complex microparticles encapsulating a *Bifidobacterium lactis* Bb12 probiotic strain: microparticle characterization and effect on viability of encapsulated probiotic cells**

BY

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**Submitted in partial fulfilment of the requirements for the degree**

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## Declaration

I declare that the dissertation ‘Poly-(vinylpyrrolidone)-poly-(vinylacetate-co-crotonic acid) (PVP: PVAc-CA) interpolymer complex microparticles encapsulating a Bifidobacterium lactis Bb12 probiotic strain: microparticle characterization and effect on viability of encapsulated probiotic cells”, which I hereby submit for the degree Magister Scientiae at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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## PUBLICATIONS AND CONFERENCE CONTRIBUTIONS

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### Conference papers

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## LIST OF ABBREVIATIONS

- AAD: Antibiotic-associated diarrhoea
- CD: Crohn's disease
- CLSM: Confocal Laser Scanning Microscopy
- DGGE: Denaturing Gradient Gel Electrophoresis
- DNA: Deoxyribonucleic acid
- EE%: Encapsulation Efficiency percentage
- EMA: Ethidium monoazide
- FDA: Food and Drug Administration
- FOS: Fructooligosaccharide
- GALT: Gut-associated Lymphoid Tissue
- GIT: Gastrointestinal tract
- GRAS: Generally Regarded/Recognised as Safe
- IBD: Inflammatory Bowel Disease
- IBS: Irritable Bowel Syndrome
- IL: Interleukin
- IFN: Interferon
- LAB: Lactic acid bacteria
- MRS: De Man Rogosa Sharpe
- NDO: Non-digestible oligosaccharide
- NEC: Necrotising enterocolitis
- PET: Poly (ethylene terephthalate)
- PGSS: Particles from Gas Saturated Solution
- PI: Propidium iodide
- PVP: Poly (vinyl pyrrolidone)
- PVAc-CA: Poly-(vinyl acetate co-crotonic acid)
- PCR: Polymerase chain reaction

PMA: Propidium azide

RESS: Rapid Expansion of Supercritical Solutions

rRNA: Ribosomal ribonucleic acid

RDP: Ribosomal Database Project

scCO<sub>2</sub>: Supercritical carbon dioxide

SCFA: Short-chain fatty acid

SEM: Scanning Electron Microscopy

SGF: Simulated Gastric Fluid

SIF: Simulated Intestinal Fluid

TNF: Tumour Necrosis Factor

UC: Ulcerative Colitis

VLDL: Very Low-Density Lipoproteins

TGGE: Temperature Gradient Gel Electrophoresis

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## SUMMARY

Microorganisms have been known to play a major role in human health since early times. The ingestion of microorganisms as probiotics to restore and/or maintain health is a widely accepted and common practice. The challenge in industry is to ensure viability of probiotics until their ingestion to their site of action, the colon, for health benefits to be realised. Microencapsulation is one of the techniques used to protect probiotic bacteria and ensure viability. A method that does not involve the use of extreme temperatures and/or solvents which would otherwise adversely affect viable cells was developed and patented. The method is solventless and is based on complexation of Food and Drug Administration-approved polymers, poly (vinylpyrrolidone) and poly (vinylacetate-co-crotonic acid) in supercritical carbon dioxide.

The use of this method of encapsulation was found to be suitable in target release in earlier studies. Microparticles produced were found to have pH-dependent swellability, protecting bioactives, in this case probiotic bifidobacteria, in acid (simulated gastric acid) and only releasing them in an alkaline environment (simulated intestinal fluid). Further studies were, however, needed to investigate the suitability of the microparticles for food and pharmaceutical applications. The current study therefore aimed to characterize these microparticles in terms of size range, distribution of bacteria within the microparticles, and particle size distribution. The average size of the *Bifidobacterium lactis* Bb12-encapsulating microparticles was found to be within the acceptable size in food applications. High encapsulation efficiency was obtained, with live bacteria distributed evenly within the microparticles, demonstrating the potential of the microparticles to deliver high numbers of probiotic cultures as required for this type of microorganisms to deliver purported benefits to the consumer.

Probiotic products are normally kept under refrigerated storage, yet the viability of bacterial cells still decreases. An additional benefit of encapsulation within microparticles would be protection of the encapsulated probiotics from the detrimental factors to which the probiotic products are exposed during storage. In order to investigate this for the microparticles in this study, the shelf life of encapsulated *B. lactis* Bb 12 powder stored in glass vials was investigated. High temperatures were used for accelerated shelf life studies. Encapsulated *B. lactis* Bb 12 maintained the viable levels above the therapeutic minimum for the duration of the study (12 weeks), which was 7 weeks more than was the case with unencapsulated

probiotic. Thus the microparticles provided protection to the probiotic cultures at temperatures much higher than those normally used for storage of probiotic products. These results further indicate the possibility for storage of the *B. lactis* Bb12 encapsulated in the tested microparticles, at ambient temperatures for at least two months, without drastic loss of culture viability.

Research has recently focused on the development of probiotic foods other than dairy and dairy-based foods. This has been necessitated by increasing vegetarian lifestyle and concerns of allergenicity. A maize-based traditional fermented beverage, *mageu*, was investigated for use as a vehicle for probiotic delivery. Although no significant difference was noted between survival of encapsulated and unencapsulated probiotic was noted, pH decrease in *mageu* with encapsulated *B. lactis* Bb 12 was less than with unencapsulated cells. This suggested that encapsulation would ensure that metabolites produced by encapsulated probiotics, if any, would not negatively affect a product in which they are incorporated. Further studies may be needed for investigation of the effect of the encapsulating microparticles in traditional fermented non-dairy products, using more acid-sensitive probiotic strains as the test strain used in the current study is well-known for its inherent resistance to acidity.

This study filled gaps in knowledge in terms of the characteristics of microparticles produced using supercritical technology. The main highlights of the research findings were that the microparticles were suitable for food applications, improved probiotic viability under non-refrigerated temperatures, and delayed browning of the probiotic powder and minimized drop in pH of the fermented product containing the probiotic encapsulated within. The results showed that microparticles encapsulating *B. lactis* Bb 12 are appropriate to consumers in areas where refrigeration is absent. Furthermore, the study showed that *mageu* is a suitable alternative vehicle to dairy-based products, for delivery of probiotic *B. lactis* Bb 12. This possibility extends accessibility of probiotic products to consumers who do not take dairy products for various reasons. There is also a potential increase of probiotic products on the market.

## INTRODUCTION

The first stages of the evolution of the use of microbes to restore health are attributed to Metchnikoff in 1907. He recommended the ingestion and absorption of large quantities of microbes. He postulated that there were many useful microbes, of which the lactobacilli hold an important place (Fuller, 1992). Research in subsequent years proved that indigenous microbes provided a mucosa-associated shield which protects the host from infectious diseases (Dobrogosz *et al.*, 2010). Interest in the use of natural methods with less or no side effects for improvement of health has also increased over the years. Probiotics are live non-pathogenic organisms, which, when ingested in sufficient amounts confer a health benefit to the consumer. The consumption of probiotics is associated with health benefits such as modulation of the immune system, prevention and/or management of acute gastroenteritis and rotavirus diarrhoea, antibiotic-associated diarrhoea and intestinal inflammatory disorders such as Crohn's disease and pouchitis, and paediatric atopic disorders (Mack *et al.*, 1999; Steer *et al.*, 2000; Fooks and Gibson, 2002). The most commonly used probiotic bacteria are those belonging to the genera *Lactobacillus* and *Bifidobacterium*, which are indigenous to the human gastrointestinal tract (GIT) (Tannock, 2001; Bielecka *et al.*, 2002). Bacteria of human origin are preferred as probiotic strains because they have been found to be the only strains that can adhere to and temporarily colonize the human GIT (Huis in't Veld *et al.*, 1994). Colonization is when a bacterial population establishes itself in size over time with no need for its introduction from an external source either through oral intake or other means.

Scepticism on the health potential of probiotics has been documented. This has been caused by poor quality and statistical ineffectiveness of marketed probiotics (Barrow, 1992; Freter, 1992, Fuller, 1994). Commercial exploitation of the probiotic concept also increased the scepticism. This has been warranted by some products on the market which are unreliable in their content, and lack clinical backing. There is need for more, large, randomised, double blind placebo-controlled studies to substantiate claims made on the effectiveness of probiotics. Even within strains of the same species, probiotic effects of bacteria may differ depending on specific capabilities and enzymatic activities. Various studies have shown that "a rather high percentage of probiotic products suffered from incorrect labelling and yielded low bacterial counts" (Fasoli *et al.*, 2003; Temmerman *et al.*, 2003; Huff, 2004; Reid, 2005). Some products have claimed the presence of bacteria that are absent in a product or those which do not exist (Sanders *et al.*, 2001, Reid, 2005).

Probiotics have traditionally been mainly incorporated into dairy products such as milk powders, yoghurt, soft-, semi-hard and hard cheeses and ice cream (Dinakar and Mistry, 1994; Stanton *et al.*, 2001; Desmond *et al.*, 2005; Stanton *et al.*, 2005; Anal and Singh, 2007). Such foods are termed ‘functional foods’, which describes “foods claimed to have a positive effect on health”. However, delivery of probiotics is no longer limited to dairy products. Increased consumer preference for non-dairy products has led to development of products such as drinks and supplements in the form of tablets, capsules and freeze-dried preparations (Rivera-Espinoza and Gallardo-Navarro, 2010).

Essentially, bioproducts, with probiotic foods being part of these products, should contain a satisfactory number of active cells at the time of consumption. A minimum of  $10^7$  viable cells per gram or millilitre of food product is needed for probiotic cultures to exert a beneficial effect (Ishibashi and Shimamura, 1993). However, probiotics do not have a long shelf life in their active form. Refrigeration is required in most cases to maintain shelf life as high temperatures can destroy probiotics cultures (Saxelin *et al.*, 1999). High oxygen levels, moisture and direct light also have adverse effects on them. Various researchers have studied and tried microencapsulation as a method for protection of probiotic cultures from the mentioned detrimental factors (Adhikari, 2000; Lee, 2000; O’Riordan *et al.*, 2001). Microencapsulation involves entrapping living microbial cells within a semi-permeable polymeric gel structure (Moslemy *et al.*, 2002). The main reasons for encapsulation are to increase the survival of bacterial cultures during processing and storage, and also for targeted delivery in the GIT. These target-delivery systems should ensure that bioactives encapsulated, in this case the probiotic strains, are not released early in the upper gastrointestinal area where bile salts and hydrolytic enzymes can adversely affect them, but are released in the intestine (Playne, 1994). Encapsulation segregates the cells from adverse environments thus potentially reducing cell injury.

In the food industry, encapsulation has many applications such as controlling of the oxidative reactions, masking flavours, colours or odours, stabilizing the core material, extending shelf life and protection of components against nutritional loss (Anal and Singh, 2007). Several studies have shown successful encapsulation and coating of bacteria using various materials and methods. The documented microencapsulation methods include spray-drying, spray-congealing, fluidised bed coating/air suspension, extrusion, coacervation/phase separation and electrostatic techniques (Anal and Singh, 2007). The use of water or organic solvents

employed by some of these techniques may compromise survival of encapsulated cells, as they are sensitive to high temperatures, moisture and solvents.

Moolman *et al.* (2006) first reported a novel encapsulation method that uses neither water nor solvents. This method is based on interpolymer complex formation between poly (vinyl pyrrolidone) (PVP) and poly (vinyl acetate-co-crotonic acid) (PVAc-CA) in supercritical carbon dioxide ( $\text{scCO}_2$ ). These two hydrophilic, low molecular mass polymers are individually soluble in or plasticizable by  $\text{scCO}_2$ . The interpolymer complex formed is less soluble than the individual polymers in both  $\text{scCO}_2$  and water, is biodegradable and has improved barrier properties compared to the two individual starting polymers. These features are useful in controlled release applications (Rolfes *et al.*, 2001). Controlled release techniques have been increasingly utilized in industries such as pharmaceutical and agrochemical technologies (Harris, 1981) and food technology (Champagne, 2007).

Successful encapsulation was achieved using this method and it was proved that no noticeable damage or morphological changes to the bacteria occurred (Thantsha, 2007). However, there are still gaps in knowledge on the characterization of this encapsulating matrix. Characterization refers to the use of external techniques to probe into the internal structure and properties of a material. Microcapsules should be of small diameter (< 100  $\mu\text{m}$ ) for higher stability and minimal undesirable organoleptic qualities when incorporated into foods (Picot and Lacroix, 2003).

Following the successful encapsulation of bifidobacteria within an interpolymer complex matrix of PVP and PVAc-CA in  $\text{sCO}_2$ , this research project aims to characterize the resulting microparticles for their potential use in foods and pharmaceuticals, and to investigate the viability and stability of the probiotics enclosed within these microparticles in powder form as well as when incorporated into a traditional fermented beverage, *mageu*. This study would be of significance especially in rural areas where refrigeration is often absent, should the encapsulated cultures survive under ambient temperatures. It will also extend the availability of probiotics to consumers who rather prefer non-dairy products.

The specific objectives of this study are to:

- Determine the morphology, inner appearance and particle size distribution of the encapsulated bacterial powder.
- Determine encapsulation efficiency and distribution of bifidobacteria within the microparticles.
- Evaluate the effect of the encapsulation method on the shelf-life of *Bifidobacterium lactis* Bb12 strain
- Determine the survival of the encapsulated bacteria in a traditional fermented product, mageu.

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# Chapter 1

## Literature Review

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## 1.1 The human gastrointestinal tract microbiota

The gastrointestinal tract (GIT) is made up of the oral cavity, pharynx, oesophagus, small intestine, large intestine, rectum, anal canal and anus (Thibodeau, and Patton, 2002). The mucosa (innermost layer of the gut wall) has a total surface area of up to  $300\text{ m}^2$ , making it the largest body area interacting with the environment (Collins *et al.*, 1998). The GIT harbours a vast and diverse community of microbes essential to host well-being. The human body contains ten times more microbial than eukaryotic cells, mainly due to the presence of this dense population of microbes in the GIT (Bengmark, 1998). The majority of these microbes are located within the distal region, called the colon or large intestine (Kovatcheva-Dachary *et al.*, 2009). The GIT has a critical role in the immune function via gut-associated lymphoid tissue (GALT) (Thibodeau and Patton, 2002). The main biological functions of the large intestine include waste storage and excretion, water and essential mineral absorption (Macfarlane and McBain, 1999).

The microbiota found in the colon plays a role in nutritive, metabolic, immunological and protective functions (Fanaro *et al.*, 2003). A complex and balanced microbial community is important for normal digestion and homeostasis in the host (Tannock, 1995). This community acts as a first line of defence against pathogens or other harmful elements ingested with food (Isolauri *et al.*, 2004). The GIT is germ-free at birth. Microbes that populate it come from the outside (Ley *et al.*, 2006). These microbes are introduced mainly from the mother and the environment. Establishment of a microbiota in the infant is not necessarily a succession, but a complex process influenced by host and microbial interactions as well as internal and external factors. The climax intestinal flora is attained in successive stages (Fanaro *et al.*, 2003). Competition for nutrients and physical space will determine the population level of each species (Adlerberth, 1999).

Colonisation of the colon by microbes is affected by a number of factors. Mode and type of delivery, maternal microbiota, bacterial load of the environment (Mackie *et al.*, 1999), sanitary conditions (Lundquist *et al.*, 1985), feeding type, and geographical location, may all influence the colonisation pattern (Guarner and Malagelada, 2003). Environmental differences between home birth versus hospitalised delivery, and in developed versus developing countries also have a bearing on the colonisation pattern. Routine hygiene conditions in hospitals reduce the amount of microbes present (Fanaro *et al.*, 2003). Colonisation starts

immediately for full-term babies delivered via the birth canal compared to those who are born by Caesarean section (Favier *et al.*, 2002). During passage through the vaginal canal the infant is introduced to the maternal microbes through ingestion of the vaginal fluids (Adlerberth, 1999). Breast milk creates an environment favouring growth of bifidobacteria. Breast-fed babies have bifidobacteria in abundance after a week and later they acquire a host of other bacteria from supplements (Fanaro *et al.*, 2003). Formula-fed babies have members of *Enterobacteriaceae*, *Clostridium*, *Streptococcus*, *Bacteroides* and then later *Bifidobacterium* species (Yuhara *et al.*, 1983). Even in some formula-fed infants bifidobacteria predominate, though in lower numbers and frequency compared to breast-fed infants of the same age-group (Klessen *et al.*, 1995; Langhendries *et al.*, 1995).

Changes in diet during the first two years of life bring about intestinal bacterial succession (Edwards and Parrett, 2002). Streptococci and members of the *Enterobacteriaceae* family such as *Escherichia coli* were proposed to be the first colonisers, followed by the strict anaerobes, bifidobacteria and bacteroides (Yoshioka *et al.*, 1991). Members of the Gram-negative genera *Bacteroides* are the most abundant, constituting about 30% of the total faecal microbiota (Gibson and Roberfroid, 1995). The population of bifidobacteria decreases with age until they become the third most dominant after the genera *Bacteroides* and *Eubacterium* (Finegold *et al.*, 1983). Other facultative and obligate anaerobes found in the gut include *Enterobacter*, *Enterococcus*, *Klebsiella*, *Lactobacillus*, *Proteus*, *Clostridium*, *Peptococcus*, *Peptostreptococcus*, and *Ruminococcus* (Vaughan *et al.*, 2000), constituting >99% of faecal bacteria (Ramakrishna, 2007). The facultative anaerobes mediate a reduction in redox potential in the lumen, making the site favourable for subsequent colonization by obligate anaerobes (Stark and Lee, 1982). Once established, the microbiota is believed to remain generally unchanged through life.

Four microhabitats have been identified within the intestinal tract. These are epithelial cell surface, intestinal lumen, the crypts and the mucous gel layer (Freter, 1992). Microbial interactions are a major factor in regulating the indigenous bacterial community (Fanaro *et al.*, 2003). Due to the huge and diverse bacterial community, bacteria have to develop means to colonize and establish themselves, either by colonization or implantation. The different bacterial populations and their niches are shown in Fig. 1.1.

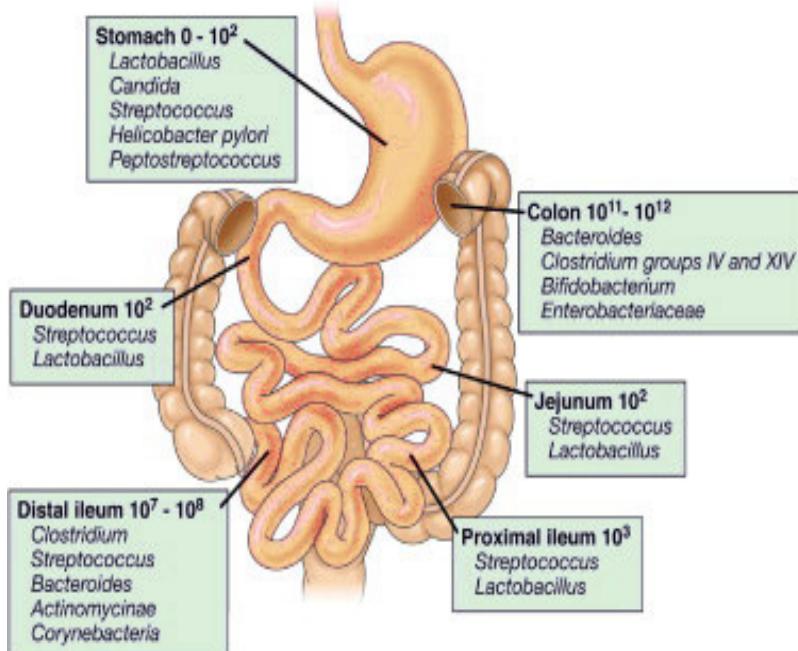


Fig. 1.1 Microbial colonisation of the gastrointestinal tract (Sartor, 2008)

The degree of microbial colonization varies throughout the length of the GIT, as shown in Fig 1.1 (Hentges, 1993; Ramakrishna, 2007, Sartor, 2008). The upper part of the GIT (stomach) usually has  $<10^2$  bacterial cells per ml because of the presence of acidic pH (bile) and pancreatic secretions, which kill most bacteria (Guarner and Malagelada, 2003). In this part, aciduric Gram-positive bacteria of the genera *Lactobacillus* and *Streptococcus* dominate. Mucosal adherence also gives these groups a competitive edge over pathogens (Kos *et al.*, 2003). The population here is further reduced by the short retention time of gastric contents and phasic propulsive motor action of epithelia towards the luminal end (Guarner and Malagelada, 2003; Macfarlane and Macfarlane, 2004). Numbers increase up to  $>10^8$  per ml in the distal ileum (Macfarlane and Cummings, 1991). The large intestine is the main site for permanent colonization (Tuohy *et al.*, 2003). The colon typically harbours  $10^{11}$ - $10^{12}$  diverse bacterial cells per gram faeces, comprised of between 300 and 500 different species (Finegold *et al.*, 1983; Cummings *et al.*, 1993; Simon and Gorbach, 1984; Simon and Gorbach, 1986). The high numbers of microbes in the colon are due to the near-neutral pH, slow transit time (approximately 4-6 hours) and substrate availability. Adherence to epithelial cells is essential for some bacteria to establish themselves in the colon (Fanaro *et al.*, 2003). Studies carried out by McCartney *et al.* (1996) on faecal samples showed obligate anaerobes (*Bacteroides* and *Bifidobacterium*) are stable in composition.

### 1.1.1 Products of metabolism by gut microflora

The microbiota present in any part of the GIT is influenced by the type of sugar present. The majority of human intestinal bacteria are saccharolytic. In the upper GIT relatively simple sugars are found. *Lactobacillus* species are found in abundance there (Vaughan *et al.*, 2005). The lower parts of the colon contain complex sugars which bifidobacteria can ferment. Ryan *et al.* (2006) noted that not all bifidobacteria hydrolyse plant-derived polysaccharides such as starch and amylopectin. However, certain species such as *B. breve* and *B. adolescentis* have these sugar-degrading abilities.

Gases, including H<sub>2</sub>, CO<sub>2</sub>, CH<sub>4</sub> and short-chain fatty acids (SCFAs) such as butyrate, propionate and acetate are produced through metabolic activities of GIT microflora (Gibson and Roberfroid, 1995; Guarner and Malagelada, 2003). Butyrate is the most important among these, and is consumed by the colonic epithelium for energy (Cummings *et al.*, 1987). It also plays a role in mucosal functions such as reducing oxidative stress and inhibiting inflammation and carcinogenesis (Hamer *et al.*, 2007). Propionate and acetate are both found in portal blood. Propionate is eventually metabolised by the liver and acetate by peripheral tissues, particularly muscle (Cummings and Englyst, 1987). SCFAs may minimise occurrence of diarrhoea through rapid absorption by the colonic mucosa, which facilitates water absorption (Cummings and Macfarlane, 1991). They also stimulate the growth of beneficial bacteria such as bifidobacteria and lactobacilli (Le Leu *et al.*, 2005; Kovatcheva-Datchary *et al.*, 2009).

Other significant products of saccharolytic fermentation include lactate, succinate, formate, valerate, caproate and ethanol (Mountzouris *et al.*, 2002). Active fermentation leads to a decrease in luminal pH, which aids in the control of certain enzymes such as amino acid decarboxylase and bile acid 7- $\alpha$ -dehydroxylase (Cummings and Englyst, 1987). Amino acid decarboxylase helps produce dopamine and serotonin from other molecules and is essential in the normal functioning of the nervous system (Buck and Ferger, 2008). Bile acid 7- $\alpha$ -dehydroxylase regulates bile acid metabolism. Gut flora can also control bile acid pool composition. Malfunctions in bile acid metabolism resulting in excessive bile salt deconjugation may lead to promotion of colon cancer and cholesterol gallstone disease in some patients (Bayerdorffer *et al.*, 1993; Berr *et al.*, 1996; Bernstein *et al.*, 2005; Ridlon *et al.*, 2010).

### 1.1.2 The role of indigenous bacteria

The health state of an individual is dependent upon the balance between the beneficial and harmful effects of the intestinal microflora (Percival, 1997). Thus, the indigenous bacteria of the human GIT are important in human nutrition and health. They assist in promoting the supply of nutrients, preventing colonization by pathogens and maintaining normal mucosal immunity (Marcfarlane and Cummings, 1999; Hooper and Gordon, 2001; Hooper *et al.*, 2002). The intestinal microbiota also influences the absorption and distribution of body fat (Backhed *et al.*, 2005). Amaral *et al.* (2008) showed that intestinal microbiota play a fundamental role in perception of pain on the skin which helps adaptability of an individual to a stressful environment and avoid tissue damage. Microbial metabolism accounts for about 50% of the daily requirement of energy available to colonocytes during fermentation of carbohydrates (Tuohy *et al.*, 2003). Colonocytes are epithelial cells lining the colon.

## 1.2 Lactic Acid bacteria

The term lactic acid bacteria (LAB) refers to a broad group of Gram-positive, fastidious, acid-tolerant, catalase-negative, non-sporing rods and cocci, which are usually non-motile (Aguirre and Collins, 1993; Vasiljevic and Shah, 2008). These microorganisms are associated with meat, plant and dairy products (Collins and Lyne, 1984). LAB are important in giving flavour, texture and preservative properties to various fermented foods (Klaenhammer, 1995; Vasiljevic and Shah, 2008). They are obligate fermenters that utilize carbohydrates for energy, producing lactic acid as the main end product (Schleifer *et al.*, 1995). Anaerobic conditions are preferred for fermentation, though some are aerotolerant (Vasiljevic and Shah, 2008). This group consists of *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc* and *Pediococcus* (Adams and Nicolaides, 1997; Anal and Harjinder, 2007).

Schlegel (1986) grouped a number of LAB according to their fermentation end products and the way they metabolise carbohydrates as their main source of energy, as either homofermentative or heterofermentative. The homofermentative group of the LAB is able to ferment glucose more directly to lactic acid than the heterofermenters (Jay, 1986). Genera such as *Lactococcus*, *Pediococcus*, *Enterococcus*, *Streptococcus* and some lactobacilli are found in the homofermenters group. Heterofermenters such as *Leuconostoc*, *Weisella* and some lactobacilli lack a respiratory chain and therefore produce energy not by oxidative

phosphorylation, but through substrate level phosphorylation (Yamazaki *et al.*, 1998). Their products of glucose metabolism are equimolar amounts of lactate, CO<sub>2</sub>, ethanol or acetate (Vasiljevic and Shah, 2008).

### 1.3 Bifidobacteria

Bifidobacteria were first described by Tissier in 1899, after isolating them from faeces of breast-fed infants (Doleires and Lacroix, 2005), where they constitute more than 95% of the total population (Yildirim *et al.*, 1999). He termed the isolated microorganisms *Bacillus bifidus communis* because of their bifid morphology (Tissier, 1900; Sidarenka *et al.*, 2008). However, a Danish microbiologist by the name Orla-Jensen proposed classification of these bacteria as *Bifidobacterium*. He believed this genus was unique and formed the link between LAB and propionic bacteria (Orla-Jensen, 1924; Sidarenka *et al.*, 2008). There was not much consensus regarding this genus and during most part of the 20<sup>th</sup> century they were still classified as LAB due to their rod-shaped morphology and fermentation pattern. Though bifidobacteria share certain biochemical and physiological properties with members of the LAB group, they are not related to them as such (Sgorbati *et al.*, 1995; Miyake *et al.*, 1998). A lot of controversy arose in relation to deciding on the phylogenetic position of some of these species (Mayer *et al.*, 2007). Increase in studies and knowledge on unique metabolic capabilities, DNA hybridisation and G+C content led to recognition of this genus (Leahy *et al.*, 2005). They are grouped in the *Actinomycetes* branch of LAB for practical and traditional reasons (Stiles and Holzapfel, 1997). The actinomycete branch has 5 genera: *Bifidobacterium*, *Brevibacterium*, *Corynebacterium*, *Microbacterium* and *Propionibacterium*.

Bifidobacteria are classed within the high G+C cluster, in contrast to other members of the LAB genera, varying from 55 to 67 mol% DNA (Vasiljevic and Shah, 2008). Other members have low G+C content (< 55 mol% DNA) and are in the *Clostridium* branch (Vasiljevic and Shah, 2008). The optimum temperature for bifidobacterial growth is 37-41°C, with a minimum between 25-28°C and maximum between 43-45°C. Their optimum pH for growth is 6.5 –7.0, with no growth at 4.5-5.0 and 8.0 –8.5 (Olsen *et al.*, 1994). They are classed as Gram-positive, non-motile, catalase-negative, non-sporeforming, obligate anaerobes (a few strains can tolerate oxygen) (Simpson *et al.*, 2004). They are pleomorphic fermentative rods, often Y-shaped or ‘bifid’ in form (Janet *et al.*, 2003) as shown in Fig. 1.2. The culture

medium used appears to affect the formation of V, X and Y shapes noted in the *Bifidobacterium* genus.

The morphology of bifidobacterial cells can be affected by their respective media, growth and culture conditions. This makes identification by phenotypic appearance unreliable (Scardovi, 1984; Bonaparte and Reuter, 1997, Mayer *et al.*, 2007). The use of gas-liquid chromatography to determine the final products of glucose metabolism is a reliable method of differentiating bifidobacteria from other related genera (Biavati *et al.*, 1992). They do not produce butyrate (Campbell *et al.*, 1997; Djouzi and Andrieux, 1997). Bifidobacteria are characterized by the presence of fructose-6-phosphate phosphoketolase. The only other genus that is characterized by this key enzyme of carbohydrate metabolism is the phylogenetically close *Gardnerella* (Gavini *et al.*, 1996; Sidarenka *et al.*, 2008).

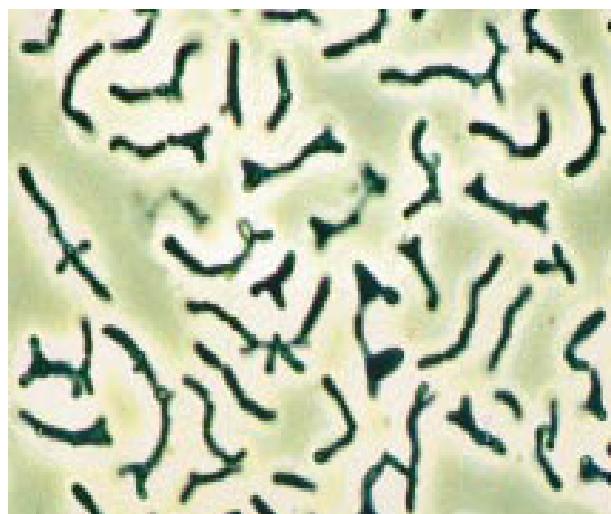


Fig. 1.2 Gram-stained *Bifidobacterium adolescentis* showing different pleomorphic shapes (Macfarlane and Cummings, 1999)

Bifidobacteria are found in abundance in the human GIT, the oral cavity and the vagina (Verhelst *et al.*, 2005; Okamoto *et al.*, 2008). More than 30 recognised species of this genus *Bifidobacterium* were isolated from the intestines of humans and some animals (Mikkelsen, 2003). Apart from intestines of humans and animals, bifidobacteria have been isolated from other sources. Species such as *B. minimum* and *B. subtile* have been isolated from sewage and *B. asteroides*, *B. indicum* and *B. coryneforme* from insects' guts (Biavati *et al.*, 1982; Ventura *et al.*, 2007). Killer *et al.* (2009) recently isolated bifidobacteria from the intestine of bumblebees.

Bifidobacteria degrade hexoses exclusively and specifically by the phosphoketolase route as described by Scardovi and Trovatelli (1974). They are characterized by the presence of glucose-6-phosphoketolase (F6PPK- E.C 4.1.2.22), a key enzyme in the heterofermentative pathway where fructose-6-phosphate is split into C<sub>2</sub> and C<sub>4</sub> moieties (Scardovi, 1986). The initial glucose degradation is entirely through the pentose phosphate pathway via glucose-5-phosphate to xylulose-5-phosphate (Fig. 1.3).

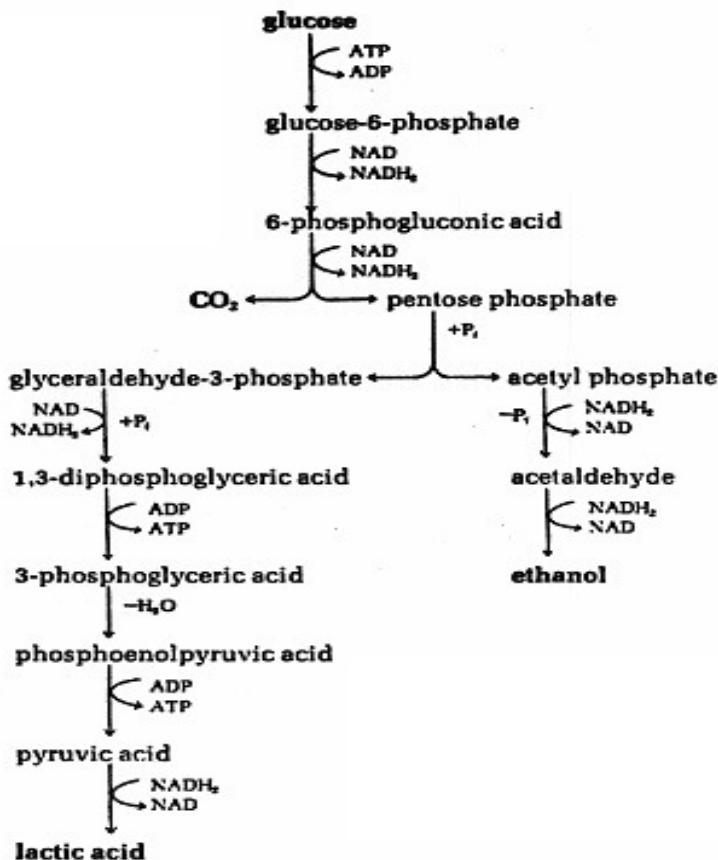


Fig. 1.3. The phosphoketolase route for bifidobacterial carbohydrate fermentation. (Whelan, 1975)

Their pathway differs from that of other heterofermenters in that they do not produce carbon dioxide (Scardovi, 1986; Sidarenka *et al.*, 2008). The xylulose-5-phosphate is thereafter cleaved to glyceraldehyde-3-phosphate and acetyl phosphate. Glyceraldehyde-3-phosphate is then reduced to lactic acid via pyruvate while acetyl phosphate is degraded via acetyl CoA and acetaldehyde to ethanol (Cloete and Atlas, 2006). The fermentation of two moles of hexose by this pathway yields three moles of acetate and two moles of lactate as follows:



*Bifidobacterium* species have been extensively studied and are attributed to have beneficial effects such as inhibition of pathogenic bacteria, immunomodulation, improved calcium absorption, inhibition of tumour formation, improved intestinal balance and lowered blood ammonia and cholesterol levels (Gibson *et al.*, 1996, Yaeshima *et al.*, 1996). Cheikhyossef *et al.* (2008) recently studied and characterized bacteriocins produced by *Bifidobacterium* spp. that have potent antimicrobial properties towards closely related bacteria, mostly pathogens. Bifidobacteria contain genes that are predicted to encode proteins that form cell envelope-associated structures, which may play a role in bacterial-host interactions. Perez *et al.* (1998) reported a 21.3kb long DNA region in *B. breve* UCC2003 that encompasses at least 17 putative exopolysaccharide (EPS) biosynthetic determinants. The role of EPS in non-pathogenic bacteria is not fully understood. However, it may aid in bacterial adherence to host cells and also allow colonic bacteria to withstand stomach acids and bile salts (Perez *et al.*, 1998). Additional antimicrobial components produced by bifidobacteria and LAB include organic acids, carbon dioxide, diacetyl, hydrogen peroxide and low molecular weight antimicrobial substances such as reutin (Ouwehand and Vesterlund, 2004).

#### **1.4 Factors affecting the normal GIT microflora**

In healthy individuals the composition of intestinal flora is stable. Exogenous and endogenous factors may affect the balance between beneficial and harmful microbes in the GIT. Natural prevalence of the beneficial bacteria (LAB and bifidobacteria) in the digestive tract may decrease with age, dietary changes, travelling, lack of food and water, antibiotic consumption, radiation, drugs for treatment of tumours and/or stress (Havenaar and Huis int'Veld, 1992; Luchansky *et al.*, 1999). Surgical operations of the stomach or small intestine, anaemia, liver or kidney diseases, peristalsis and immune disorders also affect normal GIT microbial balance (Mitsuoka, 1996; Luchansky *et al.*, 1999). The effect of one or more of these disturbances may result in loss of indigenous microflora, thus creating empty adhesion sites on the intestinal epithelium. These empty sites will become available to any organism, including transient pathogenic microorganisms (Havenaar and Huis int'Veld, 1992). Occupation by pathogenic microorganisms such as *Bacteroides* predisposes the affected individual to conditions such as bowel cancer and inflammatory bowel disease. The host becomes more susceptible to infections caused by transient enteropathogens such as *Salmonella* and *Escherichia coli* (Fooks and Gibson, 2002).

Oral administration of bacteria or a well-balanced diet may restore the normal balance between beneficial and harmful bacteria. Members of the indigenous GIT, commonly strains of lactobacilli and bifidobacteria, can be isolated and administered to produce beneficial effects (Mitsuoka, 1996). These beneficial effects include displacement of harmful bacteria through competitive exclusion. Potential mechanisms of action include acidification of the medium and production of antimicrobial agents. Metabolic activities of these beneficial bacteria result in the generation of B vitamins, together with lipolytic, proteolytic and  $\beta$ -galactosidase improving tolerance to lactose (Adams and Moss, 2000; Losada and Olleros, 2002). They also improve digestion and hence absorption of various nutrients (Adams and Moss, 2000). Other benefits include improvement in disorders such as atopic eczema, vaginitis, upper respiratory and intestinal infections (Losada and Olleros, 2002, Chapman *et al.*, 2010). These orally administered beneficial microbial cultures are collectively known as probiotics.

## 1.5 Probiotics

### 1.5.1 The concept

Fermented milk products have been used for therapeutic benefits way before knowledge of the existence of microorganisms. Consumption of these products dates back to pre-biblical times as recorded in the Bible and sacred books of Hinduism (Leahy *et al.*, 2005). The process of fermentation has been used for long-term preservation of milk, and this use can be dated back to at least 6000 years to the Fertile Crescent in the Middle East (Holzapfel, 2002; Blandino *et al.*, 2003). The traditional fermented milk products Laban Rayeb and Laban Khad were consumed in Egypt as early as 7000 BC (Vasiljevic and Shah, 2009). Some popular products such as kefir and yoghurt are claimed to have originated in the Balkans and Eastern Europe (Vasiljesic and Shah, 2008). The therapeutic benefits of consuming fermented milks are even claimed in Abraham's longevity (Kowsikoski and Mistry, 1997). The lower level of blood cholesterol in men from the African tribes of Samburu and Maasai was attributed to consumption of fermented milk products (Shaper *et al.*, 1963; Mann, 1974; Perreira and Gibson, 2002).

### 1.5.2 Definition

Metchnikoff (1907) is accredited as the pioneer of the probiotic concept in the early 1900s. The term ‘probiotic’ meaning “for life” is derived from the Greek language. The first definition of probiotics was used as a contrast to that of an antibiotic by Lilly and Stillwell in 1965 who described probiotics as “substances secreted by one microorganism which stimulate the growth of another” (Hamilton-Miller, *et al.*, 2003). Parker (1974) came up with a definition that is closer to what is used now, defining probiotics as “organisms and substances which contribute to microbial intestinal balance”. A number of years passed by and scientists were still arguing about an all-encompassing definition. Then, Havenaar and Huis In’t Veld (1992) defined probiotics acceptably 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 health effects in this host”. In the same year Fuller came up with the frequently cited definition, which was, however, more applicable to animals than humans. He defined them as “a live microbial feed supplement, which beneficially affects the host animal by improving its microbial intestinal balance” (Vilsojevic and Shah, 2008). More recently the Food and Agriculture Organisation (FAO) of the United Nations and the World Health Organisation defined probiotics as “live microorganisms (bacteria or yeasts), which when ingested in sufficient numbers confer one or more specified demonstrated health benefits for the host” (FAO/WHO, 2001). This definition may, however, need to be revised as studies have shown that both viable and nonviable bacteria can confer the same positive benefit on amelioration of colitis. This positive benefit comes as a result of the probiotic DNA, and not due to metabolites or ability to colonise the colon as previously thought. These DNA contain unmethylated nucleotides, which are ligands of toll-like receptors with a broad range of effects on mammalian innate immune system (Rachmilewitz *et al.*, 2004).

### 1.5.3 Properties of a good probiotic strain

There are some qualities that are pre-requisite for the defining of a good probiotic. Collins *et al.* (1998) made a comprehensive list that is useful as a guideline. A good probiotic should preferably be of human origin due to their demonstrated ability to adhere and colonize the human GIT (Havenaar and Huit, 1994). The probiotic bacteria should be able to transiently adhere to and colonize the epithelial cell lining so as to establish themselves in the colon (Guarner and Schaafsma, 1998; Parracho *et al.*, 2007). Adherence also guards against

flushing effects of peristaltic movements (Gupta and Garg, 2009). Probiotics should be generally regarded/recognised as safe (GRAS) (Collins *et al.*, 1998). A probiotic strain should possess a desirable antibiogram profile. It must also be genetically stable, lacking any plasmid transfer mechanism (Ziemer and Gibson, 1998). It is desirable that a good probiotic strain has technological suitability i.e. it should be inexpensively reproducible and be able to withstand stress during processing and storage, with process and product application robustness (Charteris *et al.*, 1998). A probiotic strain should not negatively affect the sensory properties of a food product into which it is incorporated (Lacroix and Yildirim, 2007). Probiotic strains have to be good vehicles for specific target delivery of peptides and recombinant proteins within the human GIT. The organism should be able to survive particularly the harsh environmental conditions of the stomach and small intestine (e.g. gastric and bile acids, digestive enzymes) (Dunne *et al.*, 2001; Parracho *et al.*, 2007). Microorganisms used in probiotic preparations should be non-pathogenic, even in immunocompromised individuals, have antimutagenic and anticarcinogenic properties and not promote inflammation in individuals (Collins *et al.*, 1998).

The most commonly used probiotic bacteria are those belonging to the genera *Lactobacillus* and *Bifidobacterium*, which are indigenous to the human GIT (Tannock, 2001, Bielecka *et al.*, 2002). These groups of bacteria are known to have no harmful effects in contrast to other gut bacteria (Kimoto-Nira *et al.*, 2007). The most recognised bifidobacteria species that are used as probiotics are *Bifidobacterium breve*, *B. animalis* subsp *lactis* formerly *B. lactis* (Masco *et al.*, 2004) and *B. longum* biotypes *infantis* and *longum* (Masco *et al.*, 2005). *Lactobacillus* strains include *L. acidophilus*, *L. rhamnosus*, *L. casei*, *L. johnsonii*, *L. gasseri*, and *L. reuteri* (Meurman and Stamatova, 2007). Species from other genera such as *Streptococcus*, *Lactococcus*, *Enterococcus*, *Leuconostoc*, *Propionibacterium*, *Pediococcus*, and the yeast *Saccharomyces* are also used in probiotic products (O'Sullivan *et al.*, 1992; Vinderola and Reinheimer, 2003; Vandenplas *et al.*, 2007; Power *et al.*, 2008). However, there are safety concerns about the use of some members of the *Enterococcus* in other countries due to the ability of this genus to transfer genes conferring antibiotic resistance (Lund and Edlund, 2001).

### 1.5.4 Probiotic foods

Probiotics can be consumed as a food component or as a non-food preparation (Stanton *et al.*, 1998). Several probiotic LAB strains are available to consumers in both traditional fermented foods and in supplemented form (Kourkoutas *et al.*, 2005). Probiotic cultures have been mainly incorporated into dairy products such as milk powders, yoghurt, soft-, semi-hard and hard cheeses and ice cream (Dinakar and Mistry, 1994; Stanton *et al.*, 2001; Desmond *et al.*, 2005; Stanton *et al.*, 2005). Dairy products offer a suitable environment for probiotic viability and growth (Ross *et al.*, 2002; Özer *et al.*, 2009), though low viability of these cultures in yoghurt has been reported (Kailasapathy and Rybka, 1997; Shah, 2000; Lourens-Hattingh and Viljoen, 2001). Probiotic bacteria in dairy fermentations play a role in preservation of the milk through production of lactic acid and other antimicrobial compounds. They also impart flavour to a product through compounds such as acetylaldehyde in yoghurt and cheese and other metabolites e.g. extracellular polysaccharides which will result in favourable organoleptic qualities to the consumer (Parvez *et al.*, 2006).

The use of other foods as vehicles for probiotics is on the increase. This is due in part to allergenicity of some consumers to milk products. Growing vegetarian alternatives have also led to soy-based probiotic foods (Farmworth *et al.*, 2007). Non-dairy products such as malt-based beverages and fruit juices (Rozada-Sanchez *et al.*, 2007; Sheehan *et al.*, 2007; Champagne and Raymond, 2008), meat, especially sausages (Ruiz-Moyano *et al.*, 2008), capsules, and freeze-dried preparations (Berni-Carnani *et al.*, 2007) have been looked at. Aragon-Alegro and colleagues (2007) investigated the idea of making a probiotic chocolate mousse and were successful.

Probiotics can be described as ‘functional foods’. This refers to foods with components (nutrient or non-nutrient) that affect function(s) in the body in a targeted way so as to have a positive effect on health (Bellisle *et al.*, 1998). Food can also be described as functional if it has a physiological or psychological effect beyond basic nutritional value (Clydesdale, 1997). Although the consumption of an average of  $10^7$  viable cells per g or ml product has been recommended for probiotics to achieve the desired health effect, administration in some situations is determined by type of strain and product (Corcoran *et al.*, 2008). For example, *L. reuteri* was effective at  $10^8$  cfu/day in reducing work place absences (Tubelius *et al.*, 2005) whilst a dose of  $1.8 \times 10^{12}$  cfu/day VSL #3 was needed to maintain antibiotic-induced

pouchitis and improve quality of life (Mimura *et al.*, 2004). The probiotic VSL#3 is a combination of 4 strains of lactobacilli (*L. casei*, *L. plantarum*, *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*), 3 strains of bifidobacteria (*B. longum*, *B. breve*, *B. infantis*) and a strain of *Streptococcus salivarius* subsp. *thermophilus* (Gionchetti *et al.*, 2006). The benefits attributed to probiotics can either be nutritional or therapeutic (Prasad *et al.*, 1998). Benefits associated are, however, strain specific (Saarela *et al.*, 2000).

### **1.5.5 Probiotic benefits**

#### **1.5.5.1 Nutritional benefits**

Microbial action in the gut, specifically by beneficial cultures, has been shown to enhance the bioavailability, quantity and digestibility of certain nutrients (Parvez *et al.*, 2006). Ingestion of probiotics is associated with improved production of riboflavin, niacin, thiamine, vitamin B<sub>6</sub>, vitamin B<sub>12</sub> and folic acid (Hargrove and Alford, 1978; Gorbach, 1997). Probiotics play a role in increasing bioavailability of calcium, iron, manganese, copper, phosphorous (Alm, 1982; McDonough *et al.*, 1983) and increase the digestibility of protein and fat in yoghurt (Fernandes *et al.*, 1987). Enzymatic hydrolysis of protein and fat leads to an increase in free amino acids and SCFAs. Organic acids such as acetate and lactate produced during fermentation by LAB lower the pH of intestinal contents thereby creating undesirable conditions for harmful bacteria (Mack *et al.*, 1999; Parvez *et al.*, 2006).

#### **1.5.5.2 Therapeutic benefits**

Patients prefer medicine with little or no side effects for treatment of their ailments. Probiotics provide such an alternative, being living, non-pathogenic organisms, which are extremely safe as indicated by their GRAS status. Probiotic bacteria are claimed to alleviate and prevent conditions such as lactose intolerance, allergies, diarrhoeal diseases, lowering of serum cholesterol, reduction of the risk associated with mutagenicity and carcinogenicity and inhibition of pathogens, as well as stimulation of the immune system (Collins and Gibson, 1999; Shah, 2007). Positive effects of probiotics are not confined to the gut only, but can extend to other parts of the body. For instance, probiotics are known to have anti-inflammatory benefit when administered parenterally (Shiel *et al.*, 2004).

### 1.5.5.2.1 Treatment and prevention of allergies

The word ‘allergy’ was coined in the 19<sup>th</sup> century from the Greek words ‘allon argon’, meaning to react differently (Smith and Frew, 2003). Allergy is also known as atopy. Individuals with this condition experience hypersensitivity to substances that are harmless to most people (Chhajer, 2005) Genetic predisposition and the environment influence the development of allergies (Matricardi, 2001; von Mutius, 2002; Kalliomaki, 2003). Substances that often cause reactions are pollen, dust mites, pet dander, insect stings and some foods (Shirakawa *et al.*, 1997; Gent *et al.*, 2009). Atopic diseases such as eczema, asthma and allergic rhinitis are chronic allergic conditions of increasing importance in developing countries (Holgate, 1999; Kalliomaki *et al.*, 2003).

Exposure to bacteria early in life has been shown to have a protective effect against allergies (Kalliomaki *et al.*, 2001). Allergic reactions may be caused by a delayed colonization with *Bifidobacterium* and *Lactobacillus* spp. in the GITs of children. There are conflicting reports about the effective use of probiotics for alleviation or prevention of allergies. *Bifidobacterium* and *Lactobacillus* strains have been shown to have potential in treating atopic eczema in children (Isolauri *et al.*, 2000; Kalliomaki *et al.*, 2001; Lee *et al.*, 2008). Administration of the probiotic *L. rhamnosus* GG to pregnant mothers for a month before delivery and later to their new-born infants caused a significant reduction in occurrence of atopic disease (atopic eczema, asthma and rhinitis) (Vanderhoof and Young, 2003). A 4-year follow-up study of the same infants suggested that the benefits extended beyond infancy (Kalliomaki *et al.*, 2003). The use of *L. rhamnosus* did not prevent neither apple nor birch pollen allergy in a group of young adults or teenagers (Helin *et al.*, 2002). Probiotics have not shown significant effectiveness in management of asthma (Vliagoftis *et al.*, 2008). These conflicting results indicate that the effects vary for different types of allergies and different probiotics. More convincing and conclusive research needs to be done on the use of probiotics in allergy treatment. Further investigations need to be carried out on which probiotics are effective at what stage in life.

### 1.5.5.2.2 Prevention and reduction of intestinal disorders

The effect of probiotics against incidence of diarrhoea is perhaps the most studied and substantiated claim to date (Reid *et al.*, 2003; Sullivan and Nord, 2005). Antibiotic-associated

diarrhoea (AAD), caused mostly by *Clostridium difficile* after antibiotic therapy, has received much attention. Antibiotic treatment causes an imbalance in the gastrointestinal ecosystem, favouring the proliferation of the indigenous *C. difficile*, which is usually present in low numbers in healthy individuals. The toxins produced by *C. difficile* cause symptoms of diarrhoea (Vasiljevic and Shah, 2008). Single or combination effects of probiotic strains of *Saccharomyces boulardii*, *L. rhamnosus GG*, *L. acidophilus* and *L. bulgaricus* have demonstrated a positive effect against AAD (Sazawal *et al.*, 2006) and diarrhoea caused by rotaviruses in children (Shornikova *et al.*, 1997a, 1997b; Vanderhoof, 2000). Enterotoxigenic *Escherichia coli* has been found to be a common aetiological agent in traveller's diarrhoea (Marteau *et al.*, 2001). The use of probiotics for treatment of traveller's diarrhoea has produced varying results depending on the species used, vehicle and dosage schedule (Black *et al.*, 1989; Hilton *et al.*, 1996). Contradictory results obtained from testing of probiotic preparations as prophylaxis treatment are probably due to methodological deficiencies, which limit the validity of their conclusions (Marteau *et al.*, 2002). The strains of *L. rhamnosus GG* and *B. animalis* Bb 12 have given the strongest evidence supporting the efficacy claims of probiotics in the treatment of acute diarrhoea in children. Administration of these strains led to reduction in the duration of the diarrhoea, shortened treatment course and reduced hospital stay for infected patients (Guandalini *et al.*, 2000).

Some infants suffer from the intestinal disorder called necrotising enterocolitis (NEC) which is caused by an extensive growth and proliferation of pathogenic bacteria (Ng *et al.*, 2009). Symptoms associated with NEC include bloody diarrhoea, abdominal distention, bilious vomiting and apnoea (periods when a baby stops breathing) (Caplan and Jilling, 2000). A combination of *L. acidophilus* and *B. infantis* was effective against the occurrence and associated mortality in pre-term babies suffering from NEC (Hoyos, 1999). A combination of probiotics in an ABC Dophilus product (consisting of *B. bifidus*, *B. infantis* and *S. thermophilus*) was shown to reduce the incidence of NEC from 16.4% in the control group to 4% in the probiotic group (Bin-Nun *et al.*, 2005).

#### **1.5.5.2.3 Prevention of inflammatory bowel disease (IBD)**

Inflammatory bowel disease (IBD) refers to a spectrum of disorders of unknown causes characterized by chronic or recurrent inflammation, ulceration and abnormal narrowing of the GIT resulting in abdominal pain, diarrhoea and gastrointestinal bleeding (Marteau 2001;

Hanauer, 2006). This disorder includes ulcerative colitis (UC), Crohn's disease (CD) and pouchitis. These conditions are grouped together but have been shown to be distinct and discrete entities (Hanauer, 2006).

Crohn's disease (CD) is predominantly a type 1 T-helper cell (Th1)-driven immune response which is characterized by excessive interleukin (IL)-12 and IL-23 production, followed by interferon (IFN)- $\gamma$  and tumor necrosis factor (TNF)- $\alpha$  (Papadakis and Targan, 2000). This affects the wall of the small intestine leading to inflammation and often granulomas (Serino *et al.*, 2009). Patients experience rectal bleeding in addition to abdominal pains and diarrhoea. They may complain of fever, fatigue, weight loss and can develop fistulae (Podolsky, 2002). Ulcerative colitis (UC) is a Th2 immune response associated with excessive production of proinflammatory cytokines including IL-5 and IL-13. UC is restricted to the colon, with chronic inflammation of the mucosa. It generally has rectal origins and spreads proximally (Papadakis and Targan, 2000; Podolsky, 2002). Sometimes patients present symptoms that make it difficult to distinguish between CD and UC. When this happens the condition is treated as indeterminant colitis, and may develop into either condition (Hanauer, 2006). Pouchitis is the most common long-term complication after pouch surgery (ileal pouch-anal anastomosis (IPAA) for ulcerative colitis (Gionchetti *et al.*, 2000) characterized by increased stool frequency and urgency, discomfort and abdominal cramping.

Individuals who suffer from IBD are predisposed to colorectal cancer later in life (Itzkowitz and Harpaz, 2004). IBD reduces patients' quality of life and the cure for this condition is unknown (Prisciandaro *et al.*, 2009). It is, however, thought that this disorder could be due to a defective mucosal barrier (Ruseler van Embden *et al.*, 1994; Sartor, 1995), resistance of T-cells to apoptosis, failure of regulatory lymphocytes and anti-inflammatory cytokines (Bamias *et al.*, 2005), or genetically susceptible individuals who have an elevated immune response to their microbiota (Sartor, 2004; Serino *et al.*, 2009).

A combination of environmental, genetic and immunological factors is also thought to play a role in the development of IBD (Danese *et al.*, 2004). This condition is notably more prevalent in developed countries, where levels of hygiene and sanitation are high compared to developing countries (Loftus and Sandborn, 2002). Optimal mucosal immune development and regulation has been proposed to be aided by early exposure to unhygienic conditions during development. Future inflammatory response will thus be prevented (Shanahan, 2004).

Smoking is also a major environmental factor influencing this condition, which interestingly has been shown to increase the chances of relapse in CD but decreasing likelihood of relapse in UC patients (Rubin and Hanauer, 2000; Danese *et al.*, 2004). Farrell and Peppercorn (2002) discovered that genetic factors may play a greater role in the incidence of CD (44-50% concordance rates between monozygotic twins) than UC (6-14%). Several studies have shown that first-degree relatives of an affected individual have a greater chance of suffering from IBD (Tysk *et al.*, 1988; Orholm *et al.*, 1991).

It has been shown that intestinal microbiota plays a role in IBD through observations that

- inflammation and lesions usually occur in regions in the GIT with a high density of microbes (ileum and colon) (Harper *et al.*, 1985; Thompson-Chagoyan, 2005)
- antibiotics can be used as a viable treatment (Sartor, 2000)
- IBD patients show a higher number of mucosa-associated bacteria compared to healthy subjects (Swidsinski *et al.*, 2002)
- intestinal inflammation can be stopped by faecal diversion but relapse can occur if flow is re-established (D'Haens *et al.*, 1998; Boirivant and Strober, 2007)
- commensal bacteria are essential for the development of the condition (Rath *et al.*, 2001).

Several studies have been conducted to investigate the efficacy of probiotics in treatment of IBD (Guandalini, 2002; Ma *et al.*, 2004; Zhang *et al.*, 2005). Although some of the results obtained were very encouraging, there is need for larger, randomised, double-blinded, placebo-controlled clinical trials to substantiate these claims. Gionchetti *et al.* (2000) carried out such a trial on forty patients using a VSL#3 probiotic. The aim was to test for the efficacy of this preparation in the maintenance of remission on chronic relapsing pouchitis. There were fewer relapses in the VSL#3 group (15%) compared to the control group (100%) in the 9-month follow-up period. The result showed that probiotics have potential in IBD prevention. In another study, *B. longum* was administered with a prebiotic (Section 1.6) Synergy 1® (Orafti, Ticuen, Belgium) an inulin oligofructose growth substrate, to UC patients. The treatment group showed significant improvement compared to the control (Furrie *et al.*, 2005). *L. rhamnosus* GG proved effective in treatment of CD in children, showing significant decrease of activity a week after commencement of therapy (Gupta *et al.*, 2000).

#### **1.5.5.2.4 Irritable bowel syndrome (IBS)**

This is a poorly understood condition believed to affect adults only, beginning in early adult life (Maxwell *et al.*, 1997; Madden and Hunter, 2002). It is typically characterized by abdominal pain, excessive flatus, variable bowel habit and bloating (Madden and Hunter, 2002). Diagnosis is difficult due to lack of positive tests and is done by exclusion (Maxwell *et al.*, 1997). Madden and Hunter (2002) suggested that IBS may be a group of distinct conditions producing similar symptoms. It has been shown that people who have suffered from acute gastroenteritis have an increased chance of getting IBS (McKendrick and Read, 1994; Thabane *et al.*, 2007). This phenomenon is known as post-infectious irritable bowel syndrome (PI-IBS) (Collins *et al.*, 2009). The causes of IBS are unknown but have been thought to include psychosocial factors (Levy *et al.*, 2004), genetic predisposition (van der Veen *et al.*, 2005), malfermentation of food residues, altered GI motility or heightened sensory function of the intestine (Camilleri, 2001). Gender would appear to be another factor as women have been found to be more susceptible to IBS than men (Madden and Hunter, 2002). All these speculations make treatment of IBS very difficult.

Several treatment methods including the use of diets, drugs, bulking agents and forms of psychotherapy such as hypnosis, have been tried. None of these treatments achieved permanent elimination of the condition (Whorwell *et al.*, 1984; Blein, 1988). Focus has now shifted towards probiotic treatment. This is based on the fact that probiotics are living non-pathogenic organisms, which are generally safe and have little or no side effects (Spiller, 2005). Alfredo (2004) demonstrated the efficacy of *Lactobacillus plantarum* LP01 and *Bifidobacterium breve* BR0 as short-term therapy for IBS. Bifidobacteria have been used successfully in various studies for the alleviation of this condition (O'Mahony *et al.*, 2005; Brenner *et al.*, 2009; Jankovic *et al.*, 2010).

#### **1.5.5.2.5 Improvement in lactose metabolism**

Lactose malabsorption is caused by a deficiency of the enzyme  $\beta$ -D-galactosidase, commonly called lactase. This enzyme cleaves lactose into its constituent components glucose and galactose, which are readily absorbed in the blood stream. Lactose intolerant individuals are described as having less than 10% of childhood levels of lactase activity (Buller and Grand, 1990). This decline, called hypolactasia, causes insufficient lactose digestion in the ileum.

Hypolactasia is indicated by high blood glucose levels or hydrogen concentration in breath upon ingestion of 50g lactose, termed lactose maldigestion (Scrimshaw and Murray, 1988). Ingestion of milk products results in ‘gastric distress’ for lactose malabsorbers, caused by hydrogen gas production by microbial action on the undigested lactose (Shah, 1993). Yoghurt and probiotic yoghurt are tolerated well by lactose malabsorbers because of the substantial amount of  $\beta$ -D-galactosidase that is found in the traditional yoghurt cultures, *L. delbrueckii* spp. *bulgaricus* and *S. thermophilus* (Shah, 2000c).

The mechanisms of action of probiotics in the treatment of various conditions are still poorly understood. Table 1.1 shows the mechanisms that have been proposed to date.

Table 1.1 Proposed mechanisms of probiotic action (Adapted from Vasiljevic and Shah, 2008)

Health effect	Mechanism	References
Prevention and treatment of IBD	Enhancement of intestinal barrier function (mucus secretion, chloride and water secretion, binding of epithelial cells by tight junction proteins). Improvement of gut permeability Alteration of immunoregulation by increasing IL-10, TGF $\beta$ , Ig A production and reduction of TNF- $\alpha$ and IFN- $\gamma$ levels Competitive exclusion Production of antimicrobial products Heat-shock protein induction (cytoprotective)	Sartor, 2004 Ng <i>et al.</i> , 2009. Madsen <i>et al.</i> , 2001 Dotan and Rachmilewitz, 2005
Inhibition of <i>H. pylori</i> , intestinal and urogenital pathogens	Antimicrobial activity (decrease luminal pH, secrete bacteriocins inhibit bacterial invasion, block bacterial adhesion to epithelial cells, rendering vital nutrients unavailable to pathogens)	Martin <i>et al.</i> , 2001 Reid, 2001 Kailasapathy and Chin, 2000
Stimulation of immune system	Recognition by toll-like receptors through induction of innate and adaptive immunity: - upregulating anti-inflammatory cytokine production - inhibiting proinflammatory cytokines and chemokines - upregulation of phagocytic activity - regulation of Th1/Th2 balance production of cytoprotective substances - prevention of cytokine-induced apoptosis	Vanderpool <i>et al.</i> , 2008
Improvement of lactose metabolism	Delivery of intracellular $\beta$ -galactosidase into human GIT and partial lactose digestion Increased viscosity of milk product (e.g. yoghurt) prolongs GIT transit time	Vesa <i>et al.</i> , 1996
Prevention of allergic diseases (atopic eczema, rhinitis)	Inhibit bacterial adhesion/translocation Immune exclusion, elimination and regulation	Vasiljevic and Shah, 2008
Prevention and reduction of Symptoms of rotavirus and antibiotic-associated diarrhoea (AAD)	Competitive exclusion Inhibit bacterial adhesion/translocation Improved immune response	Vasiljevic and Shah, 2008

## 1.6 Prebiotics

These 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 colonic bacteria and thereby improve host health (Gibson and Roberfroid, 1995). Gibson *et al.* (2004) have proposed the definition to refer to a selectively fermented ingredient that allows specific changes; both in the composition and/or activity in the gastrointestinal microflora that confers benefits to host well-being and health. This refined definition takes into account, not only the microbial changes but also the nutritional and physiological benefits attributed to prebiotics. Prebiotics can be called ‘colonic food’ because of their selective action in the colon while passing through the upper GIT unfermented (Roberfroid, 2000).

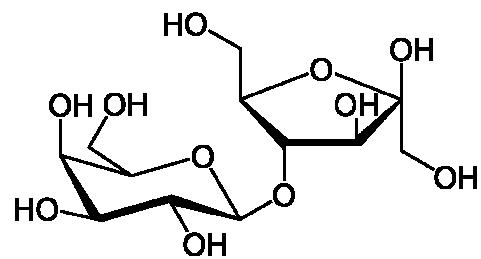
Prebiotics aim at stimulating the growth of one or more beneficial indigenous microorganisms thereby modulating the composition of the natural ecosystem compared to probiotics, which introduce exogenous bacteria into the colon (Bouhnik *et al.*, 2004). Bifidobacteria and lactobacilli are the beneficial bacteria that are almost exclusive targets of prebiotics (Gibson *et al.*, 1999; Bouhnik *et al.*, 2004). Compounds that are not digested and absorbed by the host but are preferentially fermented by *Bifidobacterium* species in the colon are called ‘bifidogenic’ factors (Shah, 2007). Prebiotics have an advantage over probiotics in that they are not affected by factors such as oxygen and heat in industrial applications. This attribute led to increased interest in their health issues (Kolita *et al.*, 2002). Some commercially available prebiotic supplements are water-soluble. Solubility in water allows their possible incorporation in any type of food and also renders them undetectable once dissolved (Douglas and Sanders, 2008). Japan produces the most probiotic and prebiotic products in the world (Shah, 2007).

### 1.6.1 Non-digestible oligosaccharides (NDOs) or non-digestible carbohydrates (NDCHs)

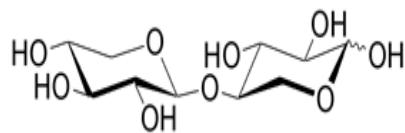
Carbohydrates can be classified according to their molecular size or degree of polymerisation into monosaccharides, oligosaccharides or polysaccharides. Monosaccharides contain one sugar unit and are the building blocks of oligosaccharides and polysaccharides. Oligosaccharides are defined as saccharides containing between 3 and 20 sugar moieties according to the IUB-IUPAC (International Union of Biochemistry- International Union of

Pure and Applied Chemistry) (Mussatto and Mancilha, 2007). Non-digestible oligosaccharides (NDO) are low molecular weight carbohydrates intermediate in nature between simple sugars and polysaccharides (Mussatto and Mancilha, 2007). Most authorities recommend that a degree of polymerisation of 10 be the division line between oligo- and polysaccharides (Cummings and Englyst, 1995). Oligosaccharides can thus be said to be short-chain polysaccharides. Oligosaccharides are water-soluble and 0.3-0.6 times as sweet as sucrose. Their sweetness depends on the degree of polymerisation and the number of mono- and disaccharides in the mixture (Crittenden *et al.*, 1996; Voragen, 1998). The anomeric carbon (C1 or C2) of the monosaccharide units of these dietary oligosaccharides make their  $\beta$ - (2→1) osidic bonds resistant to digestion by intestinal and pancreatic enzymes (Roberfroid and Slavin, 2000; Cummings *et al.*, 2001). NDOs have the capacity to increase the levels of bifidobacteria in the colon (bifidogenic effect) (Gibson and Roberfroid, 1995). Selectivity of these prebiotics is also brought about by the fact that not all colonic microorganisms have the necessary enzymes to digest them (Ziemer and Gibson, 1998).

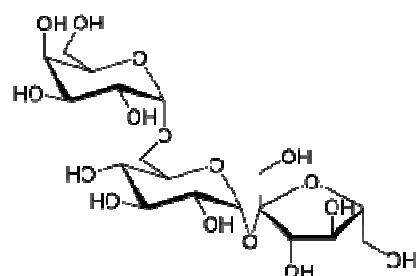
Non-digestibility can be demonstrated *in vitro* by subjecting the carbohydrates to pancreatic and small intestinal enzymes. It can be shown *in vivo* on human subjects with an ileostomy (i.e. people who have had their large intestine removed and have a stoma at the end of the ileum) (van Loo *et al.*, 1999). Principal monosaccharides in NDOs presently available are glucose, fructose, galactose and/or xylose (Sako *et al.*, 1999). Some examples of oligosaccharides are shown in Fig. 1.4.



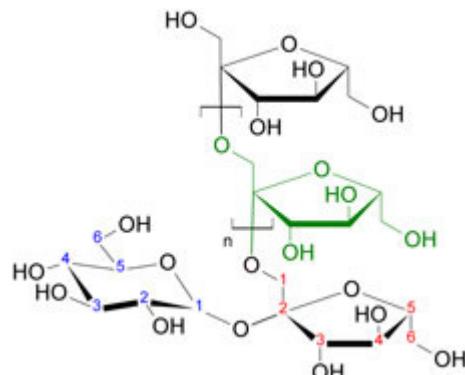
Lactulose



Xylobiose



Raffinose



Inulin

Fig. 1.4 Examples of non-digestible oligosaccharides (Neeser and German, 2004)

Classes of NDOs commercially available are cyclodextrins, fructooligosaccharides (FOS), gentiooligosaccharides, glycosylsucrose and isomaltulose (also known as palatinose). Other classes include lactulose, lactosucrose and maltooligosaccharides (Sako *et al.*, 1999).

For a carbohydrate to be classified as a prebiotic it should fulfil certain criteria. There should be selective fermentation by commensal microorganisms e.g. lactobacilli and bifidobacteria, resistance to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption. It should also stimulate the growth and/or activity of beneficial bacteria (Kolida, *et al.*, 2002). Inulin (IN) and FOS fulfil these criteria. Inulin is a linear molecule composed of  $\beta$ - (2→1) fructosyl- fructose linkages with a terminal glucose (Waterhouse and Chatterton, 1993). Inulin may contain between 2 and 60 fructose residues (Lomax and Calder, 2009). Partial enzymatic hydrolysis of inulin yields a FOS called oligofructose (OF). Oligofructose can have a chain of 2 to 8 (average of 5) fructose residues with a terminal glucose residue or a chain of 3 to 8 (average of 5) fructose residues (Roberfroid, 2005). Inulin and OF thus differ in the degree of polymerisation of the chain.

Inulin and FOS are the only NDOs that have been sufficiently studied to give adequate data to analyse their functional properties (Roberfroid, 2000). Galacto-oligosaccharides (GOS), gluco-oligosaccharides, lactulose, isomalto-oligosaccharides, raffinose, transgalacto-oligosaccharides, xylo-oligosaccharides, soya bean oligosaccharides and oat  $\beta$ -glucans are considered as prebiotic candidates (Lomax and Calder, 2009). Lactulose, which is a disaccharide, is counted among NDOs due to its bifidogenic function (Crittenden and Playne, 1996). Similarly xylobiose is included in the category of other highly polymerised xylooligosaccharides because of its technological properties and health effects though its degree of polymerisation (DP) is 2 (Vazquez *et al.*, 2000). All the prebiotic candidates except maltooligosaccharides, glycosylsucrose and cyclodextrins are reported as being bifidogenic (Ziemer and Gibson, 1998). There are conflicting views on the prebiotic classification of resistant starch. According to Shah (2004), resistant starch and non-starch oligosaccharides are not digested by some beneficial bacteria and therefore cannot be classified as prebiotics, though they are called ‘colonic food’. Douglas and Sanders (2008) disagree and mention resistant starch as prebiotic.

### 1.6.2 Sources of prebiotics

Honey, fruits (e.g. bananas) and vegetables such as chicory, garlic, barley, Jerusalem artichoke, artichoke, leeks, onion, rye, tomatoes, wheat and barley are sources of NDOs (Sangeetha *et al.*, 2005; Mussatto and Mancilha, 2007). Honey and bamboo shoots are natural sources of isomaltulose (Lina *et al.*, 2002). Raffinose and stachyose can be found in soyabeans and other leguminous seeds and pulses (Voragen, 1998). Milk is a good source of glycoproteins and oligosaccharides (lacto-N-tetraose and lacto-N-neotetraose), including those believed to be prebiotic (Petschow and Talbott, 1991; Newburg, 2000; Alander *et al.*, 2001). Human milk contains more galactooligosaccharides than cow’s milk. Oligosaccharides can be found in levels as high as (12g/l), making them the third bulkiest constituent of human milk (Newburg *et al.*, 2004).

NDOs can also be commercially produced through processes such as extraction and enzymatic hydrolysis. Enzymatic transglycosylation reactions, controlled enzymatic hydrolysis of polysaccharides and building up from simple sugars are the main ways that are employed in the commercial production of all other oligosaccharides except raffinose,

soybean oligosaccharides and lactulose (Sako *et al.*, 1999; Mussatto and Mancilha, 2007). For raffinose oligosaccharides, water, aqueous methanol or ethanol can be used for direct extraction from plant material (Johansen *et al.*, 1996). Soybean oligosaccharides are also produced by direct extraction (Mussatto and Mancilha, 2007). Lactulose is commercially produced by enzymatic action of  $\beta$ -galactosidases (E.C 3.2.1.23) on lactose. The glucose moiety is converted to a fructose residue by alkali isomerisation and the process results in a lactulose disaccharide (Villamiel *et al.*, 2002). Lactulose was incorporated into baby formulas in the 1950s but this practice was later stopped because it was not licensed and at high doses lactulose produced laxative properties (Gibson *et al.*, 2000). However, recently Riskin and colleagues (2010) have demonstrated lactulose safety in baby formula on premature infants. Their study also showed that lactulose does not cause diarrhoea when administered in small quantities. Currently, GOSS and FOSs are used in infant formulas (Parracho *et al.*, 2007; Raes *et al.*, 2009). Inulin is extracted from the plant chicory (*Cichorium intybus*) and is processed industrially to produce either short-chain fructans (oligofructose) by partial enzymatic hydrolysis using inulase (E.C 3.2.1.7) or long chain fructans by applying an industrial separation technique (Roberfroid, 2000). Sucrose can be used to manufacture commercial inulin-type fructans through transfer of fructosyl residue to and between sucrose molecules using fungal fructosyl transferases (E.C 2.4.1.9) (Cummings and Roberfroid, 1997).

Lactose is also used in the industrial production of GOS through the transgalactosylation activity of  $\beta$ -galactosidases. The lactose is purified from cow milk's whey and used in high concentration as the substrate. The main products are 4'-and 6'-galactosylactose (trisaccharides) and longer chain oligosaccharides consisting of 4 or more monosaccharides (Sako *et al.*, 1999). FOSs are manufactured from sucrose using the trans-fructosylation activity of  $\beta$ -fructofuranosidase (E.C 3.2.1.26). An alternative method for production of FOS involves the controlled enzymatic hydrolysis of the oligosaccharide inulin (Crittenden and Playne, 1996). The production of FOS and GOS requires high concentrations of the starting material for efficient transglycosylation (Park and Almeida, 1991).

### 1.6.3 Health benefits of prebiotics

#### 1.6.3.1 Improvement of mineral absorption

Colonic fermentation following ingestion of prebiotics acidifies colonic contents by increasing the concentration of short-chain carboxylic acids. They also raise the concentration of ions, especially  $Mg^{2+}$  and  $Ca^{2+}$ . This high concentration of ions favours passive diffusion, and thus aid in colonic absorption of minerals, particularly  $Mg^{2+}$  and  $Ca^{2+}$ . Some NDOs may help in mineral absorption by creating an osmotic effect whereby water enters the colon thus increasing fluid in which minerals can dissolve (Roberfroid, 2000). Most of the studies on prebiotic effects on bone development have been done on animal models, particularly rats (Scholz-Ahrens, 2007). *In vivo* studies carried out on humans showed that inulin and oligofructose increase the absorption of calcium but not iron, zinc or magnesium (Coudray *et al.*, 1997).

#### 1.6.3.2 Improvement of lipid metabolism

Feeding rats with a diet supplemented with oligofructose and inulin-type oligosaccharides significantly lowers serum triglycerols and phospholipids, but not free fatty acids. This hypotriacylglycerolaemic effect could be caused by a decrease in the concentration of plasma very low-density lipoproteins (VLDL) (Delzenne *et al.*, 1993; Fordaliso *et al.*, 1995). There are two hypotheses put forward to explain this. The first one is increased gene transcription of lipogenic enzymes caused by glucose. Dietary modulation of glucose and insulin levels has a direct effect on lipogenesis. The second hypothesis is the increase in large bowel concentration of short-chain carboxylic acids that results in a more than 2-fold increase in acetate and propionate levels in portal blood. Acetate has been reported to be a lipogenic substrate whilst propionate inhibits fatty acid synthesis (Roberfroid, 2000).

#### 1.6.3.3 Other benefits

Ingestion of prebiotics is also associated with relief of constipation due to faecal bulking and possible effects on intestinal motility, aiming at daily defecation. They suppress diarrhoea when it is associated with intestinal infections. They also reduce the risk of osteoporosis when improved bioavailability of calcium due to use of inulin-type fructans is followed by

significant increase in bone density and bone mineral content. Prebiotics reduce the risk of obesity and possibly type-2 diabetes (van Loo *et al.*, 1999).

More research needs to be done to substantiate these claims, including that of bowel cancer prevention (Ziemer and Gibson, 1996). Studies that have been carried out on animal models so far have shown promise, though human studies are required (Reddy *et al.*, 1997; Rowland *et al.*, 1998). It is vital for studies conducted *in vitro* on prebiotic effects to be carried out *in vivo* in well-designed and reproducible experiments.

## 1.7 Synbiotics

The term synbiotic is used when referring to a preparation that has a combination of a probiotic and a prebiotic. The combination could enhance the survival of the probiotic as it has a readily fermentable substrate. The prebiotic's sugar constituents and their linkages could offer protection to the probiotic against gastric acidity and proteolytic attacks by enzymes. Though this fact remains to be investigated, the mechanism could be through the coating of the probiotic and steric hindrance by the sugars. Charteris and colleagues (1998) reported the same protective mechanism on probiotics by milk proteins and mucin. Crittenden *et al.* (2006) encapsulated a *B. infantis*-FOS synbiotic within a film-forming protein-carbohydrate-oil emulsion to improve its survival and viability during non-refrigerated storage and GIT transit. Gallaher *et al.* (1996) observed a comparable synbiotic effect with oligofructose and bifidobacteria. Lactobacilli/lacitol, and bifidobacteria/GOS combinations have also been tried as synbiotics in addition to bifidobacteria/FOS (Mountzouris *et al.*, 2002). There has not been extensive study to date on synbiotic effects on prevention of allergic diseases (Johannsen and Prescott, 2009). Although further studies need to be carried out, synbiotics have been found to be effective in maintaining gut flora (Shimizu *et al.*, 2009).

## 1.8 Shelf-life of probiotics

In view of the health benefits associated with probiotics, it is not surprising that there is increasing interest in their viability. Probiotics do not have a long shelf life in their active form. Refrigeration is required in most cases to maintain shelf life as high temperatures can destroy probiotic cultures (Saxelin *et al.*, 1999). However, most probiotics still have a short shelf-life even under low temperature storage (Lee and Salminen, 1995). There is low

recovery of viable bacteria in products claiming to contain probiotic bacteria (Hamilton-Miller *et al.*, 1999; Temmerman *et al.*, 2003a).

The preservation of these probiotic microorganisms presents a challenge because they are affected by exposure to temperature, oxygen and light (Bell, 2001; Chen *et al.*, 2006). Survival of most bifidobacteria in most dairy products is poor due to low pH and/or exposure to oxygen (Gomes and Malcata, 1999). Naturally many LAB may excrete exopolysaccharides to protect themselves from harsh conditions but this is usually not enough to give them full protection (Shah, 2002). Researchers are continuously searching for ways to improve survival of probiotic cultures during processing, storage and through the harsh gastric environment. These include pre-exposing cells to sub-lethal stresses (Desmond *et al.*, 2002) and incorporation of micro-nutrients such as peptides and amino acids (Shah, 2000). Exposure to sub lethal stresses may result in significant decreases in cellular activity, cell yield and process volumetric productivity (Doleyres and Lacroix, 2005). Genetic modification of probiotic strains to cope with stress is another alternative (Sheehan *et al.*, 2006; Sheehan *et al.*, 2007; Sleator and Hill, 2007). Other methods include cell propagation in an immobilised biofilm (Doleyres *et al.*, 2004), two-step fermentations, use of oxygen-impermeable containers and microencapsulation (Özer *et al.*, 2009).

## 1.9 Microencapsulation

The terms immobilisation and encapsulation have been used interchangeably but refer to different things. Encapsulation can be defined as “the process of forming a continuous film around an inner matrix that is wholly contained within the capsule wall as a core of encapsulated material” whilst immobilisation refers to the trapping of material within or throughout a matrix (Vidhyalakshmi *et al.*, 2009). Microencapsulation refers to a technology of packaging solids, liquids or gases in miniature, sealed capsules that can release their contents at controlled rates under influences of specified conditions (Anal *et al.*, 2006; Anal and Singh, 2007). It separates cells from their environment until release under specified conditions. The main reasons for encapsulation of probiotic cultures are to increase the survival of these cultures during processing and storage, and also for targeted delivery in the GIT. The nature of the matrix/wall is such that it allows small molecules to pass in and out, and strong enough to retain the bacterial cells and minimise phage contamination (Dembczynski and Jankowski, 2002). Nutrients and metabolites can diffuse through the semi-

permeable membrane easily (Franjione and Vasishtha, 1995; Jankowski *et al.*, 1997; Gibbs *et al.*, 1999). The small diameter of the microcapsule helps to reduce mass transfer limitations and encourage uniform cell growth (Dembczynski and Jankowski, 2002; Vidhyalakshmi *et al.*, 2009). Release of the encapsulated core is caused either by mechanical rupture of the cell wall, fracture by heat, solvation, or diffusion through the wall (Franjione and Vasishtha, 1995; Brannon-Peppas, 1997).

Microspheres are important in the food, agrochemical and pharmaceutical industries for target release of bioactives. Encapsulation has various uses in the food industry which include controlling the oxidative reaction, stabilising the core, ensuring sustained or controlled release (both temporal and time-controlled release) and protection against nutrient loss. It also improves shelf life, and masks unfavourable odours, colours or flavours (Anal and Singh, 2007).

There are different ways to manufacture microcapsules in the pharmaceutical and food industries. The physical methods include pan coating, air-suspension coating, centrifugal extrusion, vibrational nozzle and spray drying (Anal and Singh, 2007). The chemical methods include interfacial polymerisation, *in-situ* polymerisation and matrix polymerisation (Vidhyalakshmi *et al.*, 2009). Various biopolymer systems that have been used to encapsulate probiotics are discussed below. The advantages of encapsulating probiotics in biodegradable polymer systems are that

- Cells are easier to handle in core compared to suspension or slurry
- Cells can be quantified allowing for dosage control
- Cryo- or osmoprotectants can be incorporated to improve survival of bacteria
- A further coat can be applied once the microcapsule has dried (Anal and Singh, 2007).

## 1.9.1 Systems used for encapsulation of probiotics

### 1.9.1.1 Alginate systems

Alginic acid is a polyuronic acid extracted from seaweeds. It is composed of various proportions of 1-4 linked  $\beta$ -D-mannuronic and  $\alpha$ -L-guluronic acids. There is instantaneous polymerisation on addition of sodium alginate to a calcium solution (Anal *et al.*, 2003). Alginate is non-toxic to cells and is an accepted food additive (Sheu and Marshall, 1993). Various researchers have used alginate in microencapsulation (Sultana *et al.*, 2000; Dembczynski and Jankowski, 2002; Hansen *et al.*, 2002). Chandramouli *et al.* (2004) used this method to encapsulate *Lactobacillus* spp. They observed that cell viability increased as gel concentration and microcapsule size was increased. This can only be done up to a certain size as too large a size (1-3mm) has an adverse effect on sensorial properties of enriched foods (Sultana *et al.*, 2000; Hansen *et al.*, 2002).

Chan and Zhang (2002) developed a direct compression encapsulation method using methacrylic acid as an enteric coating on lyophilised *L. acidophilus*. The coating material was a mixture of sodium alginate and hydroxypropyl cellulose in the ratio 9:1. This method has potential as a way of protecting cells during storage in gastric transit as it is relatively easy to use and does not involve any liquid. Tests done *in vitro* showed release of probiotic would be at the end of ileum and beginning of colon. Mechanism of release was thought to be due to erosion of the alginate gel layer. Its major drawback is that excessive pressure (> 90 MPa) causes loss of cell viability.

### 1.9.1.2 Protein and polysaccharide mixtures

Gelatin is a thermally reversible gelling agent and because of its amphoteric nature, can be incorporated with anionic-gel forming polysaccharides such as gellan gum (Anal and Singh, 2007). A mixture of alginate, pectin and whey protein was used to encapsulate *Bifidobacterium* cells (Guerin *et al.*, 2003). Protection conferred by extra membranes (protective sheaths) formed from encapsulating material was compared to those without. The extra membranes were formed by conjugating whey protein and pectin. Survival of free and encapsulated *B. bifidum* in simulated gastric and bile acid conditions was investigated and it

was noted that after 1 hr of incubation in acid (pH 2.5), there was 4.75 log reduction of free cells compared with <1 log encapsulated cells. Free cells in 2 and 4% bile salt solution showed greater mortality (4-7 log) compared to membrane-bound cells (2-3 log). Cells in double membrane showed less mortality (<2 log) because of enhanced protection to acidic conditions and high bile salt concentrations.

### **1.9.1.3 Encapsulation in $\kappa$ -carrageenan**

Carrageenan is a common food additive that occurs naturally and is extracted from marine macroalgae. The cell slurry is added to heat-sterilised carrageenan solution at around 40-45°C. Cooling the solution to room temperature causes gelation. Beads are formed by dropping the cell-polymer mixture into KCl solution (Anal and Singh, 2007). However, KCl solution was reported to be inhibitory to some organisms such as *Streptococcus thermophilus* and *L. bulgaricus* (Audet *et al.*, 1988). Locust bean gum was later combined with  $\kappa$ -carrageenan to encapsulate LAB to enhance their survival during production (Audet *et al.*, 1990; Audet *et al.*, 1991). It was found that mixing  $\kappa$ -carrageenan with locust bean gum in the ratio 2:1 had a synergistic effect in strengthening the gel beads.

### **1.9.1.4 Rennet-gelled protein encapsulation**

Rennet is used in foods as a food-approved enzyme. It is a protein that has functional properties that can be used to control microcapsule size. Rennet is a proteolytic enzyme complex that offers an alternative to producing small, water-insoluble microcapsules compared to alginate beads. Using rennet-gelation of skim milk concentrates, Heidibach and colleagues (2009) found that there was higher survival of encapsulated *Lactobacillus paracasei* and *Bifidobacterium lactis*. This improved survival was attributed to the buffering capacity of the protein matrix that created a higher local pH within the matrix. Encapsulated cells are thus protected from extreme acidic conditions in the human stomach, which would otherwise decrease their numbers (Shah, 2000). This process also has the advantage of using low temperatures, as there is instant gelation of the beads between 18 - 20°C (cold-set gelation).

### 1.9.1.5 Interpolymer encapsulation in supercritical carbon dioxide

Moolman *et al.* (2006) recently reported an encapsulation method that uses neither water nor solvents. This method is based on interpolymer complex formation between poly (vinyl pyrrolidone) (PVP) and poly (vinyl acetate-co-crotonic acid) (pVAc-CA) in supercritical carbon dioxide ( $\text{scCO}_2$ ). A substance reaches a supercritical state at temperature and pressure above its critical point. The substance is neither a gas nor a liquid but possesses properties of both, making it unique. Supercritical fluids combine liquid-like properties with gas-like transport properties (Moshashaee *et al.*, 2000). Manipulation of pressure and/or temperature can be done to change the density of the fluid. Since supercritical fluids have a wide spectrum of solvent characteristics, they can be used as solvents in different techniques (Frederiksen *et al.*, 1997). Supercritical technology has been used successfully in the food, perfume, oil and pharmaceutical industries (Fredereksen *et al.*, 1997; Brunner, 2005; Franceschi *et al.*, 2008).

PVP and PVAc-CA are low molecular weight polymers approved for pharmaceutical use by the US Food and Drug Administration (FDA) (Thantsha *et al.*, 2009). These two hydrophilic, low molecular mass polymers are individually soluble in or plasticisable by  $\text{scCO}_2$ . The interpolymer complex formed is less soluble than the individual polymers in both  $\text{scCO}_2$  and water, and is biodegradable. It also has improved barrier properties compared to the two individual starting polymers (Vidhyalakshmi *et al.*, 2009).  $\text{ScCO}_2$  is an environmentally benign gas with low reactivity parameters and low critical parameters ( $T_c = 31.1^\circ\text{C}$  and  $P_c = 73.8$  bar). Further advantages of  $\text{CO}_2$  are that it is non-flammable, non-toxic, inexpensive, recyclable and generally regarded as safe (Subramanian *et al.*, 1997). *B. longum* Bb46, *B. lactis* Bb12 and indomethacin were encapsulated by this method (Thantsha *et al.*, 2009).

### 1.10 Probiotic culture detection and viability assessment

It is imperative that during the shelf-life of a probiotic product there be microbes in adequate amounts to be beneficial to the consumer. Proper labelling of the microorganisms in a product and their amounts is important. There are various techniques used to test for bacterial viability in probiotic products. These can be culture-dependent or culture independent. Some of these methods are discussed below.

### 1.10.1 Viable counts

Standard plate counting is a culture-dependent method used for estimating the number of viable cells. Selective media are used to cultivate the desired microorganisms. Portions of samples are homogenised or blended before being serially diluted in an appropriate diluent, then plated in or onto a suitable agar medium. The plates are incubated at an appropriate temperature for a given time. Visible colonies are counted and the number of colony forming units in the original sample calculated, taking into account the dilution factor (Jay, 1992). This method has been criticised for underestimation of counts due to bacteria forming chains and/or clumping (Auty *et al.*, 2001). Four terms have been used to describe different stages of microorganisms: viable (active and readily culturable), dormant (inactive but ultimately culturable), active but unculturable and dead (inactive and unculturable) (Kell *et al.*, 1998; Kramer *et al.*, 2009). Isolation media used may be insufficiently selective affecting the reproducibility of results (Roy, 2001). Though plate counting is arduous and time consuming, no method has yet been found that completely replaces it. It is still being routinely used in assessing viability of probiotic cultures in various foods, often in conjunction with culture-independent methods (Temmerman *et al.*, 2003b; Masco *et al.*, 2005; Lopez-Rubio *et al.*, 2009).

### 1.10.2 Molecular detection

Molecular approaches have played a significant role in accelerating identification of microorganisms. They are reproducible and give more reliable and accurate results than physiological-biochemical methods (Sidarenka *et al.*, 2009). The major advantage of molecular methods is their universality: the same methods can be applied to genomes or separate genes of different groups of microorganisms and also for the characterization of both cultured and uncultured organisms (Farber, 1996). A number of molecular techniques are available and some of them are briefly described here.

#### 1.10.2.1 Polymerase chain reaction

The polymerase chain reaction (PCR) is a technique that allows for analysis of short sequences of DNA or RNA. A single or few copies of a particular sequence are amplified to

generate thousands or millions of copies. A number of buffers, primers, nucleotides, enzymes and nucleic acid to be amplified are placed in the PCR reaction vessel (Schochetman *et al.*, 1988). The principle of PCR relies on three processes (i) denaturation of double stranded DNA (ii) annealing of primers, and (iii) primer extension. Highly conserved genes such as the 16S rRNA gene have been proposed for identification purposes. In addition to this gene, others such as *recA* (a major enzyme involved in recombination), *tuf* (encoding elongation factor Tu), and *Idh* (coding for L-lactate dehydrogenase) have been proposed for differentiating between closely related bifidobacteria (Kullen *et al.*, 1997; Roy and Sirois, 2000; Ventura *et al.*, 2003).

The study of ecosystems in a particular habitat has been made easier over the years with the establishment of community profiles (Schmidt *et al.*, 1991). PCR-amplified 16S rRNA fragments are cloned, sequenced and compared to sequences available in the rRNA databases such as GenBank, EMBL and Ribosomal Database Project (RDP) (Maidak *et al.*, 2001). Extreme caution however needs to be taken in the use of these public sequence databases e.g. GenBank as some of the information is unreliable or poorly documented (Vankerckhoven *et al.*, 2008).

### **1.10.2.2 Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE)**

Improvements on basic PCR and electrophoresis to enhance specificity and speed led to the development of the techniques denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) (Singh *et al.*, 2009). In DGGE, DNA fragments of the same length but different sequences can be separated based on the decreased mobility of their partially melted double stranded molecules on polyacrylamide gel with denaturing agent (formamide or urea) (Ercolini, 2004; Singh *et al.*, 2009). TGGE uses the same principle, but separation is dependent on a temperature gradient. DGGE and TGGE are very effective molecular tools with high discriminatory power; even single nucleotide differences can be picked up (Singh *et al.*, 2009). Masco *et al* (2005) detected two bifidobacterial species in a product by DGGE analysis whereas cultivation on selective media failed to do so.

### 1.10.2.3 DNA-intercalating agents

Treatment with DNA-binding dyes and subsequent PCR analysis uses membrane integrity as the criterion in determining viability of cells. Live cells are able to exclude DNA-binding dyes such as ethidium monoazide (EMA) and propidium monoazide (PMA), while dead cells or those whose membrane integrity has been compromised are able to pick up these stains (Kramer *et al.*, 2009). These dyes form covalent bonds with DNA upon exposure to visible bright light and thus inhibit subsequent PCR amplification. Only DNA from live cells with intact membranes is selectively amplified (Nocker *et al.*, 2009).

### 1.10.2.4 Quantitative real-time PCR

Quantitative real-time PCR is another method for bacterial enumeration. However, real-time PCR does not distinguish between DNA arising from live or dead cells, thus DNA from dead cells contributes to the result (Kramer *et al.*, 2009). This problem can be solved by using dyes discussed above. This will ensure that only DNA from live cells is amplified and thus quantified. A standard curve is used in association with real-time PCR. Standard plate counting is used to obtain these values.

### 1.10.2.5 Pulsed-field gel electrophoresis

In pulsed-field gel electrophoresis (PFGE) the DNA fragments are separated in a continuously reorienting electric field according to a predetermined programme (Satokari *et al.*, 2003). Although PFGE is considered as the ‘gold standard’ in genetic typing showing strain-specific profiles distinctly (Farber, 1996), Masco *et al.* (2005) calls for caution in interpretation of results. The limitations of PFGE are that it requires special devices that are not easily available in many microbiology and biochemical laboratories, is labour-intensive and has a limit to the number of samples that can be screened at a time (Singh *et al.*, 2009).

### 1.10.2.6 Fluorescent labels

Viable cells can be detected and enumerated by use of fluorescent labels. The commercially available LIVE/DEAD® *BacLight™* viability and counting kit (Molecular Probes) has increased in popularity in various fields (Abreu *et al.*, 2006; Berney *et al.*, 2007; Gonzalez-Pimentel *et al.*, 2009; Lee *et al.*, 2009). The kit consists of two nucleic acid stains SYTO® 9 and propidium iodide (PI). Green-fluorescent SYTO9 (excitation and emission maxima, 480 and 500 nm, respectively) penetrates both viable and nonviable cells. Red-fluorescent propidium iodide (excitation and emission maxima, 490 and 635 nm, respectively) penetrates cells with damaged cell membranes (Auty *et al.*, 2001). Assessment under the microscope will distinguish ‘live’ (green-stained) from ‘dead’ (red-stained) cells (Berney *et al.*, 2007). Conventional epifluorescence microscopy may be used for viewing stained cells in liquid samples such as milk (Bunthof and Abbe, 2002). Confocal laser scanning microscopy (CLSM) has also been used (Palencia *et al.*, 2008). The high-resolution technique of CLSM has increased sensitivity and reduced out-of-focus blur (Berney *et al.*, 2007). Rapid enumeration of the bacteria can be achieved through image analysis (Caldwell *et al.*, 1992).

Flow cytometry is a rapid and sensitive technique that measures each cell individually (Bunthoff and Abbe, 2002). Physiological characteristics such as membrane integrity, enzyme activity, respiration, membrane potential and intracellular pH can be tested for using flow cytometry. A number of viability probes are available for use in addition to the LIVE/DEAD® *BacLight™* kit. Bunthoff and colleagues (2002) used carboxyfluorescein diacetate (cFDA) to stain intact cells with enzymatic activity, TOTO-1 for cells with damaged membranes and SYTO 9 for total cell enumeration. Doherty *et al.* (2009) used the BD Cell Viability assay kit (BD Biosciences, Oxford, UK), which contains the stains thiazole orange and propidium iodide.

Fluorescent in situ hybridization (FISH) allows for the identification of specific bacterial groups in a population without cultivation. Generally, 16S rRNA probes that are 15-25 nucleotides in length are labelled with a fluorescent dye at the 5' end, and are used to hybridise cells that have been fixed with appropriate chemicals under stringent conditions. Flow cytometry or epifluorescence microscopy can be used to view stained cells after stringent washing (Wagner *et al.*, 2003). However, it has been noted that not all microbial

cells can be permeabilised by oligonucleotide probes using standard fixation protocols (Amman *et al.*, 1995). The design of nucleotide probes requires prior sequence information. This can be a problem in the identification of previously ‘undetected’ groups (Favier *et al.*, 2002). In addition, this technique is dependent on accessibility of the target and the number of ribosomes per cell (Zoetendal *et al.*, 2004). The use of one labelled oligonucleotide usually gives low signal intensity. Stoecker *et al.* (2010) developed a double labelling of probes method (DOPE-FISH) to counter this.

## 1.11 Conclusions

Probiotics have several health benefits to the consumer when ingested in sufficient amounts. Most probiotics are ingested orally. The challenge that is faced is their survival in sufficient numbers from processing, storage and gastric transit until they reach the colon. Encapsulation is a method gaining in popularity to protect probiotics until targeted release. The use of supercritical fluids in encapsulation is a method that seeks to overcome challenges of solvent use and extremes of temperatures associated with other methods, which are detrimental to cell viability. Viability is essential in probiotic functionality. The level and viability of probiotic cells contained in a probiotic product determine its quality. Often the number of viable cells decreases before consumption, even in refrigerated products. Rapid methods of determining the quality of probiotic products are needed and are also being developed. The LIVE/DEAD *BacLight* kit has been used in conjunction with a microplate fluorochrome assay for rapid evaluation of probiotic viability. Traditionally plate counting has been used for enumeration. The method has long incubation periods and is often cited as underestimating viable numbers when compared to other methods. Despite these shortcomings, plate counting has still maintained its position in bacterial enumeration. It is still widely used because no method has yet been developed to replace it. It functions as reference in standard curves for other methods considered to be more advanced such as quantitative real-time PCR and FISH. Therefore plate counting will be used for enumeration in this study. The microplate fluorochrome assay will also be used.

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# Chapter 2

## Characterization of the poly-(vinylpyrrolidone)-poly-(vinylacetate-co-crotonic acid) (PVP: PVAc-CA) interpolymer complex matrix microparticles encapsulating a *Bifidobacterium lactis* Bb12 probiotic strain

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## 2.1 Abstract

The method of producing poly-(vinylpyrrolidone)-poly-(vinylacetate-co-crotonic acid) (PVP: PVAc-CA) interpolymer complex matrix microparticles in supercritical carbon dioxide ( $\text{scCO}_2$ ), encapsulating bacteria, has been recently developed. This study was aimed at probing the external and internal structure of these microparticles. The encapsulation efficiency and distribution of encapsulated *Bifidobacterium lactis* Bb12 within these microparticles were also investigated. Scanning electron microscopy (SEM) revealed irregular, mostly small, smooth microparticles with no visible bacterial cells on the surface. However, some of the microparticles appeared to have porous surfaces. An average particle size of 166.1  $\mu\text{m}$  was obtained. Both SEM and confocal laser scanning microscopy (CLSM) showed a high density of bacterial cells within the microparticles. An average encapsulation efficiency of 96 % was achieved. Thus, the microparticles have the potential to be distributed evenly in foods, deliver adequate amounts of probiotics and produce minimal adverse effects on the texture and mouthfeel of the foods into which they are incorporated.

**Keywords:** Probiotic, *Bifidobacterium lactis* Bb12, Microencapsulation, Supercritical  $\text{CO}_2$  Interpolymer complex, Poly-(vinylpyrrolidone)-poly-(vinylacetateco-crotonic acid)

## 2.2 Introduction

The ingestion of live cultures of good bacteria (probiotics) is associated with health benefits such as alleviation or treatment of inflammatory bowel disease, lactose intolerance, vaginosis and allergies (Reid *et al.*, 2003; Reid *et al.*, 2004; Fujimori *et al.*, 2007; Parra and Martinez, 2007; Roessler *et al.*, 2008; Stamatova and Meurman, 2009). Strains of the lactic acid producing bacteria, lactobacilli and bifidobacteria, are mainly used as probiotics (Fuller, 1991). Bifidobacteria are anaerobic, fastidious microorganisms. Their survival in commercial products has been reported to be difficult (Varnam and Sutherland, 1994; Kailasapathy and Chin, 2000; Ding and Shah, 2009). Probiotic microorganisms have to be protected to prolong their survival during manufacture, storage, gastric transit until they reach the colon.

Microencapsulation is one of the techniques used to protect them. Encapsulation involves entrapping living microbial cells within a semi-permeable polymeric gel structure (Moslemy *et al.*, 2002). Materials commonly used for encapsulation are polysaccharides derived from plants (starch and its derivatives), seaweed (alginic,  $\kappa$ - carageenan) or bacteria (gellan, xanthan) and animal proteins (milk, gelatin) (Rokka and Rantamaki, 2010).

Two major problems to be considered and overcome in selecting an encapsulation method on probiotics are size and delivery of live microbes. Size is important because when probiotic powder is incorporated into food vehicles there should be minimal negative effect on sensory feel. Probiotic bacteria are typically 1-5  $\mu\text{m}$ , thus nanotechnology techniques are excluded. Ideally, microparticles should be <350  $\mu\text{m}$  in diameter (Robitaille *et al.*, 1999). A desirable microencapsulation method should ensure that bioactivity of a substance is not lost or altered during manufacturing and storage. It should also protect the core until its release at its site of action, in targeted delivery. At least four encapsulation methods have been used on probiotics. These are extrusion, emulsion, spray-coating and spray-drying (Anal and Singh, 2007).

Extrusion is a popular method of encapsulation because of its ease, simplicity and low cost. Probiotic bacteria such as *B. longum*, *B. bifidum*, *B. infantis* and *L. acidophilus* have been encapsulated in alginate using this technique (Hussein and Kebary, 1999; Shah and Ravula, 2000; Lee and Heo, 2000; Petrovic *et al.*, 2007). Large bead sizes (0.1- 0.5 mm) result from extrusion. The technique also results in variable particle size distribution (Petrovic *et al.*,

2007). Slow formation of beads compared to emulsion can make this technique difficult for large-scale production (Krasaekoop *et al.*, 2003).

In the emulsion technique, a small volume of the cell-polymer suspension (discontinuous phase) is added to a large volume of vegetable oil (continuous phase) such as sunflower oil, canola oil, corn oil or soybean oil. Smaller bead sizes compared to the extrusion method are obtained, varying in range from 25 µm to 2 mm (Petrovic *et al.*, 2007). Lactic acid bacteria have been successfully encapsulated using this technique for batch (Lacroix *et al.*, 1990; Maitrot *et al.*, 1997) and continuous fermentation (Audet *et al.*, 1992; Lamboley *et al.*, 1997). Higher costs may be incurred using this technique compared to the extrusion method because of the need for vegetable oil (Krasaekoop *et al.*, 2003).

Spray-drying involves high temperatures which are detrimental to live cells, unless if they are stress-adapted (Champagne and Fustier, 2007). The major reason for loss of viability is cytoplasmic membrane damage although DNA, the cell wall and ribosomes are affected at high temperatures (Teixeira *et al.*, 1997; Petrovic *et al.*, 2007). Probiotic cells are also negatively affected by osmotic pressure, oxygen exposure and dehydration during this process (Brennan *et al.*, 1986; Teixeira *et al.*, 1997; Meng *et al.*, 2008). Spray-chilling uses the same concept as spray-drying, except that cold air is introduced to solidify the molten matrix with low melting point (32 - 42 °C) into small particles. It is considered the most inexpensive method (Gouin, 2004). However, the technique has been rarely used on probiotics. The limitation to this technique is difficulty in scaling up; current methods are only practical at laboratory scale (Champagne and Fustier, 2007).

In spray coating a solid core is kept in motion in a specially designed reaction vessel either by injection of air or by use of a rotor. The liquid coating material is sprayed in and covers the solid core, thus encapsulating it. Spray coating is viable at large scale; however, it is difficult to master. Information on how to use this technique is also proprietary, meaning a company has exclusive rights on the method and it is not free for public viewing and use (Champagne and Fustier, 2007).

Supercritical technology has recently been studied as an alternative to the methods discussed above. It has the advantage of gentle processing with minimal energy input and low negative environmental effects (Korhonen, 2002). Supercritical carbon dioxide (scCO<sub>2</sub>) has been successfully used in the extraction of desirable food components such as flavours,

antioxidants and lipids, as well as removal of undesirables such as caffeine and cholesterol (Korhonen, 2002; Novik *et al.*, 2006; Park *et al.*, 2007; Grosso *et al.*, 2008; Hou *et al.*, 2010).

The use of scCO<sub>2</sub> has also been used in encapsulating pharmaceutical preparations and more recently probiotic bacteria (Moolman *et al.*, 2006; Shinde *et al.*, 2010). Supercritical fluid coating is a solventless method which uses low or no heat. This is a major advantage in microencapsulation of living bacterial cells which may be negatively affected by the use of organic solvents and are thermolabile. In addition to being toxic, organic solvents are also flammable. There is no evaporation of solvents required therefore processing time could be significantly reduced (Bose and Bogner, 2007). ScCO<sub>2</sub> is ideal for encapsulating live cells as it only dissolves the coating material and does not affect the core (Sunkara and Kompella, 2002; Bose and Bogner, 2007).

Rapid expansion of supercritical solutions (RESS) is the most common technique used in pharmaceutical preparations. The major drawback of RESS is that most families of molecules are insoluble in CO<sub>2</sub>. A possible solution could be to use other supercritical fluids such as N<sub>2</sub>O and light hydrocarbons. However, these alternative candidate fluids are usually hazardous and not as environmentally friendly as CO<sub>2</sub> (Raynie, 1993; Fages *et al.*, 2004). CO<sub>2</sub> has the advantage of being inert and recyclable. It is also odourless and tasteless therefore it will not affect the smell or taste of a product (Shinde *et al.*, 2010). The other possible solution would be to use a solvent to dissolve the material before using RESS. Low toxicity solvents such as ethanol or acetone can be used. However, the point of using this method as ‘solventless’ is lost (Fages *et al.*, 2004). The alternative technique to be considered to overcome the shortcomings of RESS is particles from gas saturated solution (PGSS).

PGSS usually uses lower temperatures and lower gas consumption compared to RESS (Weidner *et al.*, 2003; Mishima, 2008). CO<sub>2</sub> is a dense gas which can be solubilised and used as a solute (Fages *et al.*, 2004). It dissolves in polymer matrices, reduces glass transition temperature and improves processing at reduced temperatures (Moolman *et al.*, 2006). In the PGSS process a reactor is loaded with a dry blend of polymers together with the bioactives. The reaction vessel is pressurised with scCO<sub>2</sub> resulting in the plasticisation of the polymers to form a gas-saturated solution. A helical blender is used to mix the contents before they are depressurised into a product chamber through a nozzle. The combination of cooling and escaping CO<sub>2</sub> causes dispersion of fine, solid particles (Shinde *et al.*, 2010).

Moolman and colleagues used poly-(vinylpyrrolidone) (PVP) and poly-(vinyl acetate crotonic acid) (PVAc-CA) to form an interpolymer complex (Moolman *et al.*, 2006). They were the first group to report this method on microencapsulation of probiotic bacteria. The PVP: PVAc-CA microparticles enclosing a drug, indomethacin, have been previously characterized (Moolman *et al.*, 2006). However, the microparticles encapsulating bacterial cells have not been characterized as yet. It is expected that the microparticles encapsulating drugs and bacteria will differ due to the different properties of these actives. The aim of this study was therefore to characterize the PVP: PVAc-CA microparticles based on particle size and particle size distribution, their morphology, encapsulation efficiency and distribution of bacteria within the matrix.

## 2.3 Materials and methods

### 2.3.1 Bacterial cultures

*Bifidobacterium lactis* Bb12 was obtained in freeze-dried form from Chr-Hansen. The culture was stored at -20 °C and then used as freeze-dried powder in encapsulation experiments.

### 2.3.2 Bacterial encapsulation

Bacterial encapsulation was carried out using the basic interpolymer complex system as described previously (Moolman *et al.*, 2006, Thantsha *et al.*, 2009).

### 2.3.3 Determination of encapsulation efficiency

The encapsulation efficiency (EE %) was measured by determining the number of bacteria entrapped into the microcapsules. Absolute alcohol was used to dissolve the polymers to release entrapped bacterial cells. Enumeration was done using the pour plate method in De Man, Rogosa and Sharpe (MRS) medium (Merck Pty., Ltd) supplemented with 0.05 % cysteine hydrochloride. A ¼ strength solution of Ringer's solution was used to prepare serial dilutions. Plating was carried out in triplicate. The plates were incubated at 37 ° C for 72 hrs

in anaerobic jars with Anaerocult A (Merck, Germany) for indication of anaerobic condition. Encapsulation efficiency was calculated using the equation (Pourshahab *et al.*, 2011):

$$\text{EE\%} = \frac{(\text{total initial bacteria in encapsulation mix}) - (\text{released bacteria})}{(\text{total initial bacteria in encapsulation mix})} \times 100$$

### **2.3.4 Particle size analysis**

A Microtrac S3500 Series Particle Size Analyzer (Microtrac Inc.) with tri-laser technology was used in order to determine the particle size distribution of the samples. Microtrac S3500 utilizes Tri-Laser Technology for particle size measurement. Wet measurements were done for the samples using water (refractive index 1.33) as the medium for conveying the sample to the measuring cell.

### **2.3.5 Microscopy**

#### **2.3.5.1 Scanning electron microscopy**

The morphology, surface appearance and inner appearance analysis of the microcapsules was performed by scanning electron microscopy (SEM) (Pehkonen *et al.*, 2008). The encapsulated bacterial powder was coated with carbon before sputtering with gold under argon atmosphere (Emitech K550X, Ashford, UK). For viewing the distribution of the bacteria inside the capsule, the powder particles were frozen in liquid nitrogen, and then ground using pestle and mortar. The ground particles were transferred from the mortar to aluminium pins using double sided adhesive tape. Gold sputtering was done before viewing under a JSM-840 microscope (JEOL, Tokyo, Japan).

### 2.3.5.2 Confocal laser scanning microscopy

A glycerol-based staining technique using LIVE/DEAD® *BacLight*™ kit according to Auty *et al.* (2001) was done, with minor modifications. Briefly 3 µl of SYTO 9 and 1.5 µl of Propidium iodide solution were vortexed in solution. Encapsulated bacterial powder (~ 10 µg) was mixed with this solution in 10 µl of glycerol on a microscope slide. A Zeiss 510 META confocal laser-scanning microscope (Jena., Germany) was used for imaging under the 100 X magnification lens. Confocal illumination was provided by a 488 nm Ar/Kr laser.

## 2.4 Results and discussion

### 2.4.1 Particle size of the microparticles

The microparticles reported in this study were irregular, with approximate uniformity in size distribution (Table 2.1; Fig. 2.2A) and an average diameter of  $166.1 \pm 43$  µm (mean ± standard deviation, n=6). The average D10, D50 and D90 values obtained for the microparticles were 48.16, 166.06 and 382.55 µm, respectively. Taking into consideration the D50 for example, these results meant that half of the microparticles produced had a diameter less than 166 µm. The size of the *B. lactis* Bb12 cells is (1.5-2.2 µm x 0.6 µm); therefore the microparticles encapsulating this bacterium should be between 1.6 and 350 µm, or 1.6 and 100 µm, according to Robitaille *et al.* (1999) and Picot and Lacroix (2003), respectively. Thus, the average microparticle size obtained in this study was appropriate as it fell within the standard size of the microparticles for food applications according to the literature (Robitaille *et al.*, 1999). The average size distribution of the microparticles produced is shown in Fig. 2.1. Most of the microparticles were within the acceptable range of >100 µm and <1 mm. On average, only 10% of the microparticles were smaller than 100 µm. Microparticles larger than 1 mm affect the mouthfeel of the foods supplemented with them by causing coarseness (Anal and Singh, 2007) while beads smaller than 100 µm have been associated with lower encapsulation efficiencies (Zhao *et al.*, 2008), and they do not offer significant protection to the encapsulated bacteria in simulated gastric fluids (SGF) when compared to non-encapsulated cells (Anal and Singh, 2007). The PVP: PVAc-CA microparticles have high encapsulation efficiency (96%) and have previously been proven to

significantly improve viability of the encapsulated bifidobacteria in SGF (Thantsha *et al.*, 2009).

Microparticles both smaller and bigger than those in this study have been reported by researchers elsewhere. The bifidobacteria-loaded alginate poly-l-lysine microparticles with an average size of 84.8  $\mu\text{m}$  were reported (Cui *et al.*, 2006). Recently, Kasra-Kermanshahi *et al.* (2010) reported 1-2 mm chitosan-coated alginate microcapsule loaded with *Lactobacillus*. Similar microspheres with an average size of 345.43  $\mu\text{m}$  encapsulating *B. bifidum* were also reported (Chavarri *et al.*, 2010). O'Riordan *et al.* (2001) reported *Bifidobacterium* PL-1 loaded starch microparticles of 5  $\mu\text{m}$  as measured by SEM. López-Rubrio *et al.* (2009) produced poly (vinyl alcohol) electrospun fibres with a mean diameter of 150 nm, encapsulating *B. animalis* Bb12, obtained through coaxial electrospinning.

Table 2.1 Mean particle size, polydispersity index and encapsulation efficiency (EE %) of the PVP: PVAc-CA microparticles

Batch #	Mean particle size ( $\mu\text{m}$ )	EE (%)		
		D50	D10	D90
1	160.9 $\pm$ 44	37.43 $\pm$ 2.33	495.15 $\pm$ 24.68	99.4
2	173.95 $\pm$ 13	60.19 $\pm$ 18.96	314.15 $\pm$ 107.98	90.8
3	163.35 $\pm$ 85	46.89 $\pm$ 2.73	338.45 $\pm$ 74.32	99.6

Values given as average  $\pm$  standard deviation (SD), n=3

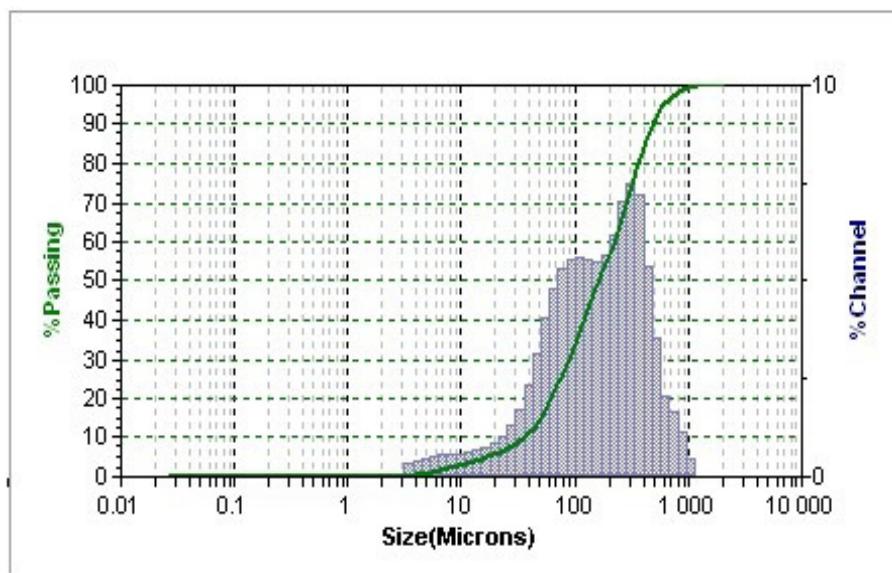


Fig. 2.1 Average size distribution of the PVP: PVAc-CA microparticles encapsulating *B. lactis* Bb12

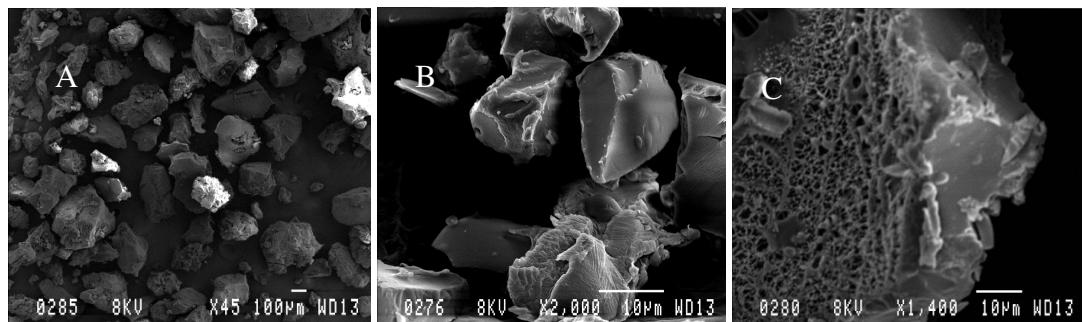


Fig. 2.2 SEM images of PVP: PVAc-CA interpolymer complex matrix microparticles encapsulating *B. lactis* Bb12 showing (A) morphology and particle size, (B) smooth surfaces and (C) a porous matrix.

#### 2.4.2 Encapsulation efficiency

Though the average size of the microparticles was small ( $166.1\text{ }\mu\text{m}$ ), there was dense loading of the probiotic strain as revealed by both SEM and CLSM (Fig 2.3). The absence of free cells in the view field (Fig. 2.2A) confirms efficient encapsulation and shows that there was uniform coating. Table 2.1 shows the encapsulation efficiencies obtained in the three

different encapsulation trials. On average, an encapsulation efficiency of 96% was obtained. Thus, the PVP: PVAc-CA microparticles will potentially deliver high numbers of the probiotics.

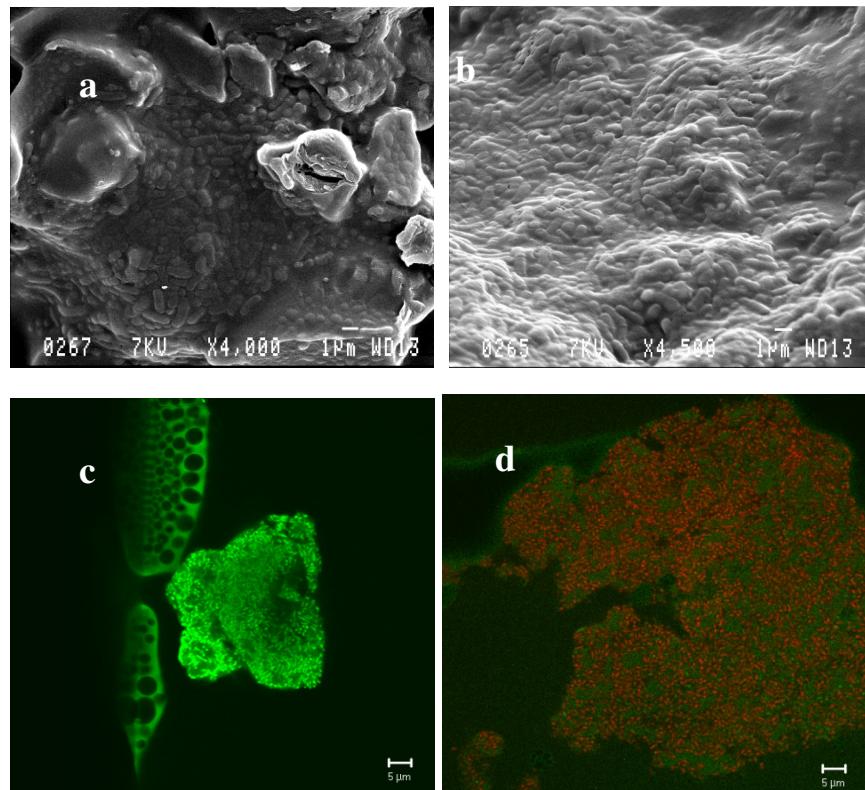


Fig. 2.3 Microscopy images showing distribution of *B. lactis* Bb12 within the PVP: PVAc-CA microparticles. a and b: SEM images, c: CLSM image of freshly prepared microparticles and d: CLSM image of microparticles stored at 30 °C for 30 days.

Fig 2.3 shows the high load of bacteria in the microcapsules. The presence of high numbers of viable bacteria also shows the non-toxicity of the encapsulation method to living cells. Moderate conditions for encapsulation using scCO<sub>2</sub> are used which do not stress the cells. CO<sub>2</sub> has low critical temperature and pressure (31 °C and 73.8 bar, respectively)

### 2.4.3 Morphology of microparticles and distribution of encapsulated bacteria

Scanning electron microscopy has been applied in the analysis of various microcapsule systems (O'Riordan *et al.*, 2001; Yang *et al.*, 2001; Donthidi *et al.*, 2010). Preparation for analysis of outer and inner structures is simple for dry powders as only coating with gold is required before mounting and viewing (Zhao *et al.*, 2008). SEM revealed as determined by visual inspection different shapes of the microparticles ranging from irregular to almost spherical microcapsules (Fig 2.3A and 2.3B). The produced microparticles were, however, not as spherical as the other microcapsules reported in the literature. This could be attributed to the rapid depressurisation process, leading to rapid CO<sub>2</sub> escape from the particles, fast solidification and fracture of the microparticles. Shape affects flowability. Most of the microparticles were almost spherical. This indicates that they will be evenly dispersed in the final products (Zhao *et al.*, 2008). The outer surfaces of most of the microcapsules were smooth (Fig. 2.2B). However, some microparticles had pores (Fig. 2.2C). The presence of pores in microcapsules has also been observed by other researchers (Sheu and Marshall, 1993; Finnie *et al.*, 2000; Sultana *et al.*, 2000). Truelstrup Hansen *et al.* (2002) believe that this porosity could be caused by presence of bacteria which can affect cross-linking of material during microcapsule formation. In this study pores might have formed due to CO<sub>2</sub> escape from the microparticles during solidification. Pores in microparticles are undesirable as they could affect the release of encapsulated cells (Ravi *et al.*, 2008). The pores were only present on few of the microparticles, thus would have negligible negative effects of release of encapsulated bacteria. To prevent release of bacterial cells some researchers resort to putting an extra coating layer to cover these pores (Ding and Shah, 2009).

Fig. 2.3 shows even distribution of bacteria within the microparticles as observed using SEM and CLSM. Mechanical sectioning of the interpolymer complex allowed for the visualisation of the internal contents using SEM (Fig. 2.3a). The use of the LIVE/DEAD Baclight kit in combination with flow cytometry, fluorometry and microscopy has increased in popularity over the years (Ericsson *et al.*, 2000; Alakomi *et al.*, 2005; Berney *et al.*, 2007). The kit contains two nucleic acid stains, the green-fluorescent SYTO 9 and the red-fluorescent propidium iodide. SYTO 9 stains both live and dead bacteria. Propidium iodide penetrates only those with compromised membrane integrity, quenching SYTO 9 fluorescence when both dyes are used (Boulos *et al.*, 1999). Auty and colleagues (2001) developed a non-destructive method that is glycerol-based. The technique allows for the viewing of probiotic

bacteria without dissolving encapsulating material. The microparticles stained using the glycerol-based dual staining with the LIVE/DEAD BacLight kit are presented in Fig. 2.3c and 2.3d. There was high loading of live bacteria in freshly prepared microparticles (Fig 2.3c) and a mixture of live and dead cells, with a high proportion of dead cells in microparticles that were stored at 30 °C for a month (Fig. 2.3d). Bifidobacteria are strict anaerobes that are affected by oxygen. This may possibly mean that although the interpolymer complex matrix protects the encapsulated probiotic bacteria due to its pH-dependent swellability as indicated previously (Thantsha *et al.*, 2009), there may be limitations with regards to its protection against oxygen penetrability over time. This has not been specifically investigated yet. However, incorporation of modifiers with good oxygen barrier properties such as glycerol monostearate to the basic system has already been investigated by the researchers in this group, and it has proven to be possible.

## 2.5. Conclusions

This study shows that PVP: PVAc-CA microparticles encapsulating *B. lactis* Bb12 probiotic strain have the desired spherical shape and size (166 µm). High loading of the encapsulated bacteria was also achieved. This has good implications for applications of the microparticles in probiotic foods as they have the potential to deliver sufficient numbers of viable bacteria due to their high encapsulation efficiency. Their shape should allow good distribution in foods while their small size will ensure minimal effects on food organoleptic properties.

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# Chapter 3

**Accelerated shelf life stability study of poly-(vinylpyrrolidone)-poly-(vinylacetate-co-crotonic acid) (PVP: PVAc-CA) encapsulated**

*Bifidobacterium lactis Bb12*

### 3.1 Abstract

Commercial probiotic production faces the challenge of retention of probiotic viability until consumption. Numbers of viable bacterial cells inevitably decrease over time. For the probiotics to be of benefit, they have to survive above the therapeutic minimum over the duration of their shelf life. Storage conditions such as temperature, humidity and water activity play a major role in how long probiotics will keep at recommended levels. Encapsulation is currently a method of interest used to protect probiotics against adverse conditions and as a means to increase the shelf life of probiotic bacteria. In this study samples of poly-(vinylpyrrolidone)-poly-(vinylacetate-co-crotonic acid) (PVP: PVAc-CA) encapsulated and unencapsulated *Bifidobacterium lactis* Bb 12 were stored in glass vials at 30 °C for 12 weeks. At weekly or fortnightly intervals the water activities of the stored samples were analysed and the viable counts were determined. Water activity was moderately low, within the acceptable range of 0.2-0.3 for most of the storage period. Encapsulated viable cell numbers decreased over the 12-week storage period but maintained levels above the recommended minimum. However, unencapsulated bacteria maintained these levels up to only 5 weeks. Encapsulation extended the viability of the enclosed bifidobacteria by 7 weeks. Unencapsulated bacteria showed more discolouration or browning compared to encapsulated bacteria. Encapsulated bifidobacteria in this study showed better survival over the study period and had delayed discolouration, therefore making their application in industry possible.

**Keywords:** Encapsulation, probiotic, water activity, bifidobacteria, *Bifidobacterium lactis* Bb12, browning

### 3.2 Introduction

Bifidobacteria are associated with healthy human intestinal tracts and have thus been used as probiotics (Meng *et al.*, 2010). Their probiotic benefits have been extensively studied (Yaeshima *et al.*, 1997; Saarela *et al.*, 2000; Ouwehand *et al.*, 2002; Abe *et al.*, 2009a). Products that contain probiotics can be grouped into three main categories (i) conventional foods such as fermented products containing probiotic bacteria; (ii) food supplements or food formulations mostly used as mode of delivery for probiotic bacteria; and (iii) dietary supplements (Fasioli *et al.*, 2003; Kramer *et al.*, 2009).

Probiotic supplements on the market can be found in capsule, liquid or powder form (Czinn and Blanchard, 2009). The nature of food matrices associated with probiotics will affect microbial growth. Liquid probiotics lose their potency within 2 weeks and therefore should be avoided. Dry products have an advantage of longer shelf-life (Weinbreck *et al.*, 2010). The challenge in the food industry would then be to preserve viability during water removal, exposure to oxygen and elevated temperatures during drying (Jankovic *et al.*, 2010).

Viability is one of the pre-requisites for probiotics to elicit full functionality (Maukonen *et al.*, 2006). An elevated water activity ( $a_w > 0.25$ ), storage temperature and the presence of atmospheric oxygen often affect cell viability (Teixeira *et al.*, 1995; Anal and Singh, 2007; Weinbreck *et al.*, 2010). It is possible that higher temperatures are incurred during transportation and storage, especially in summer or in hot countries (Abe *et al.*, 2009). Several options for ensuring maintenance of probiotic viability during drying and storage until use by consumers have been explored. These include selection of probiotic strains which are better survivors, subjecting probiotics to sub-lethal stresses, microencapsulation and use of osmo-, thermo-, and cryoprotectants during drying. Microencapsulation as an option has increased in popularity over the years (Stanton *et al.*, 2005). Encapsulation is a method used to create a microenvironment in which probiotics will survive during technological processing, storage until targeted release (Weinbreck *et al.*, 2010).

Most products on the market are kept at refrigerated storage. Even at these temperatures viability at the end of shelf-life is low. It is generally recommended that probiotic bacteria be at a minimum of  $10^6$  cfu/g or ml for them to elicit positive health benefits (Tamime *et al.*, 1995; Kailasapathy and Chin, 2000). Essentially, the levels of live bacteria should be at or above this recommended therapeutic minimum. In this study, *Bifidobacterium lactis* Bb12

was encapsulated in an interpolymer complex formed under supercritical conditions in carbon dioxide. The encapsulated probiotic powder was subjected to accelerated shelf-life studies. The powder was stored at  $30 \pm 2$  °C over a period of 12 weeks. The aim was to determine the stability of this probiotic strain during this period at a temperature much higher than the normal storage temperature for probiotic products. It would be expected that if the probiotic can survive a certain period at the elevated temperature, then it would potentially keep longer at lower or refrigerated temperatures where most probiotic products are kept.

### **3.3 Materials and methods**

#### **3.3.1 Bacterial cultures**

*Bifidobacterium lactis* Bb12 was obtained in freeze-dried form from Chr-Hansen. The culture was stored at -20 °C and then used as freeze-dried powder in encapsulation experiments.

#### **3.3.2 Bacterial encapsulation**

Bacterial encapsulation was carried out using the basic interpolymer complex system as described previously (Moolman *et al.*, 2006, Thantsha *et al.*, 2009). Two controls were prepared as follows:

1. Physical blend of freeze-dried bacteria and unprocessed polymers (PVP and pVAc-CA)
2. Physical blend of freeze-dried bacteria and PGSS-processed polymers.

#### **3.3.3 Preparation of simulated intestinal fluid (pH 6.8) for release of encapsulated *B. lactis* Bb 12**

Simulated intestinal fluid was prepared according to the US Pharmacopeia. Monobasic potassium phosphate ( $\text{KH}_2\text{PO}_4$ ) weighing 6.8 g was dissolved in 250 ml of distilled water. A

volume of 77 ml of 0.2 N NaOH followed by 500 ml of dH<sub>2</sub>O was added and the solution vortexed for 30 s. Ten grams of pancreatin were added and the pH adjusted to 6.8 with 0.2 N NaOH or 0.2 N HCl. The volume was made up to 1000 ml with dH<sub>2</sub>O. The solution was filtered through a 0.45 µm membrane.

### **3.3.4 Accelerated shelf-life studies**

#### **3.3.4.1 Storage of microparticles**

Encapsulated bacteria and controls were stored separately in polytop glass vials. The glass vials were placed in an incubator maintained at 30 ± 2 °C for 12 weeks. Subsamples were taken from these samples for analysis.

#### **3.3.4.2 Release of bacteria from encapsulating microparticles**

To determine viable counts, entrapped bacteria were released from the microparticles according to the method of Sultana *et al.* (2000) with modifications on diluent and release time. Encapsulated bacterial powder weighing 1 g was added to 9 ml simulated intestinal fluid (pH 6.8) in a stomacher bag and incubated at 37 °C for 2 h. The contents of the bag were then homogenised in a Lab-blender 400 stomacher for 30 minutes.

#### **3.3.4.3 Viable plate counts**

An aliquot volume of 0.1 ml was transferred into sterile ¼ strength Ringer's solution and 10-fold serial dilutions performed. A volume of 0.1 ml of appropriate dilutions was pour plated on De man, Rogosa and Sharpe (MRS) agar (Merck, Pty. (Ltd)), supplemented with 0.05 % cysteine hydrochloride, in triplicate. The plates were incubated at 37 °C for 72 h in anaerobic jars with anaerocult A strips (Merck, Germany) for indication of anaerobic conditions. The

numbers of colonies grown were counted and from these viable cells calculated and reported as colony forming units per gram (cfu/g).

### **3.3.5 Water activity ( $a_w$ ) measurement**

The water activity of the stored samples was measured in duplicate using the Pa<sub>w</sub>Kit handheld water activity meter (Decagon Devices, Inc., UK) according to the manufacturer's instructions. Readings were taken weekly or fortnightly over the 12-week storage period.

## **3.4 Results and discussion**

### **3.4.1 Probiotic viability during storage**

The numbers of viable probiotic bacteria in products must be available in sufficient amounts until the products reach the end of their shelf-life. However, viable bacterial cells inevitably decrease over time, presenting a challenge in commercial probiotic production. High temperatures are associated with low bacterial viability as demonstrated by Bruno and Shah (2003), with optimal viability for long-term storage achieved at – 18 °C. A temperature of 20 °C was found to be unsuitable (Bruno and Shah, 2003, Meng *et al.*, 2008). A higher temperature of 30 °C was used in this research for accelerated stability studies. If probiotics are viable at this high temperature then it would be expected that at lower temperatures, where probiotic products are normally kept, they would survive for longer. This is because survival of bacteria is generally better at lower than at higher temperature. Fig. 3.1 shows the changes in viability of encapsulated and unencapsulated *B. lactis* Bb12 over the 12 week test period.

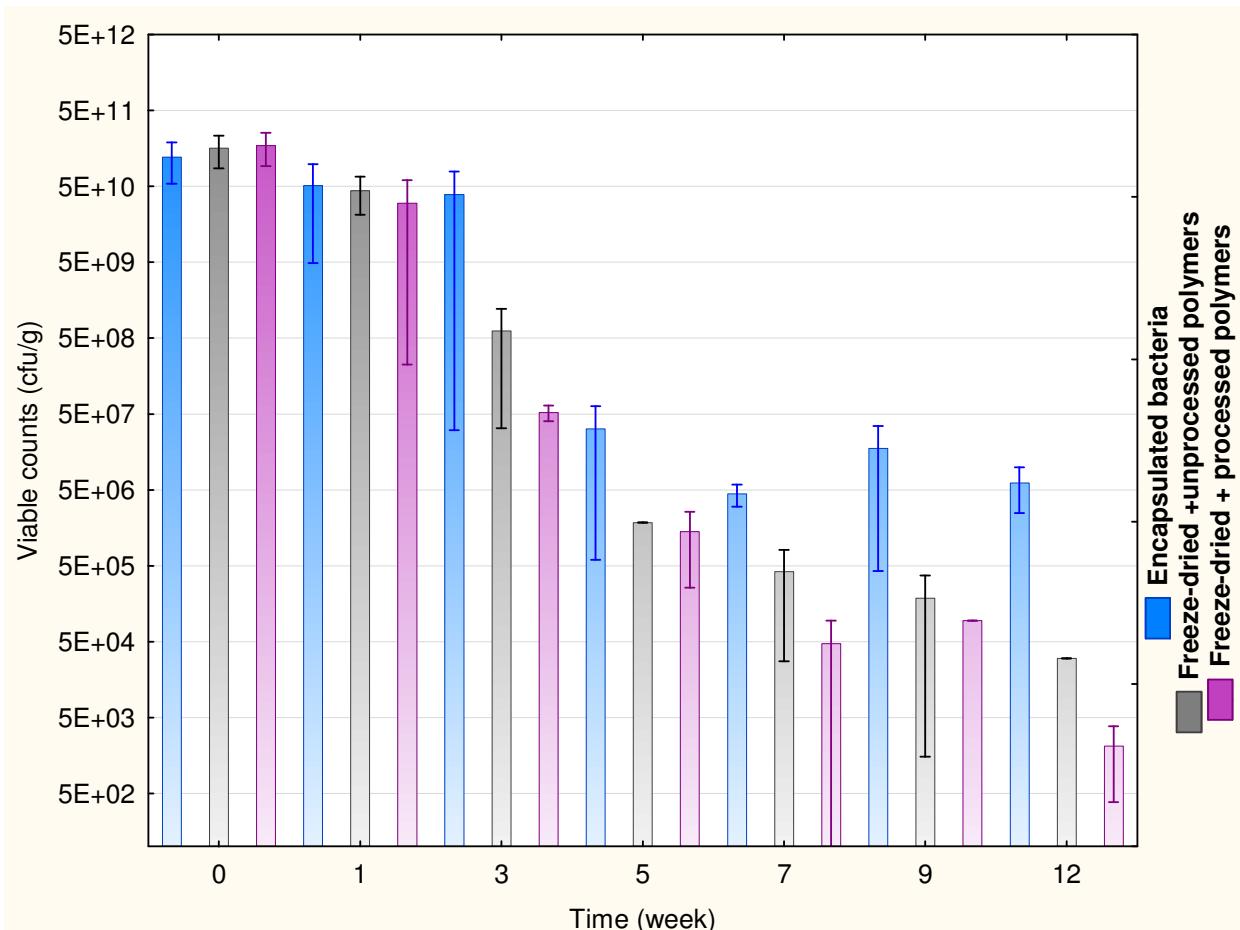


Fig 3.1 Viability of encapsulated and unencapsulated *B. lactis* Bb12 stored at 30°C for 12-weeks

Throughout the study higher viable counts were obtained for encapsulated bacteria compared to the unencapsulated controls (Fig. 3.1). There was a 0.05 log<sub>10</sub> decrease in the number of viable cells in the encapsulated sample over the first week. Unencapsulated bacteria experienced a 1 log<sub>10</sub> reduction over the same period. The highest reduction in viable counts for encapsulated bacteria occurred between the 3<sup>rd</sup> and 5<sup>th</sup>-week period, when there was a 3.1 log<sub>10</sub> decrease in number of viable bacteria. Unencapsulated cells in control 1(unprocessed polymers + freeze-dried bacteria) and control 2 (freeze-dried bacteria + polymers processed in reactor) decreased by 2.5 and 1.5 log<sub>10</sub>,respectively. The reason for this decline could not be fully explained. However, it was noted that during this period the highest water activities were recorded (Table 3.1). Elevated water activities affect the viability of bacteria and therefore this increase in water activity could have also contributed to viability losses. Thereafter the decline was gradual, possibly due to adaptation of surviving bacteria to the conditions. Encapsulated bacteria showed a 4 log<sub>10</sub> decrease over the storage time, whereas

the control 1 and control 2 decreased by 7 and 8  $\log_{10}$  respectively. At the end of 12 weeks viable numbers of encapsulated bacteria were at  $6 \times 10^6$  cfu/g. Within the same period, unencapsulated bacteria had dropped below the recommended minimum ( $3 \times 10^4$  and  $2 \times 10^3$  cfu/g respectively, for control 1 and control 2). The controls decreased below the therapeutic minimum after 5 weeks. Encapsulation therefore increased survival of probiotics from 5 to 12 weeks.

Oxygen toxicity is another factor which affects probiotic survival. Deoxidants and dessicants improve survival of probiotic bacteria over time (Hsiao *et al.*, 2004). These researchers reported better survival of *B. longum* Bb46 cells in glass bottles as compared to those in poly (ethylene terephthalate) (PET) bottles. Their results are in agreement with the findings of Shah (2000), who successfully showed reduction of cell death in probiotics stored in glass bottles as compared to plastic containers (O'Riordan *et al.*, 2001). PET has higher oxygen permeability as compared to glass (Ishibashi and Shimamura, 1993, Hsiao *et al.*, 2004). The probiotics in this study were stored in glass vials. This could have helped in their increased survival over time. However, since the samples were not stored in a vacuum, oxygen permeation could have played a part in loss of viability over time, especially since it was not determined how permeable or not the interpolymer matrix is to oxygen.

The effect of encapsulation on the survival of probiotics over time has been studied by various researchers (Weinbreck *et al.*, 2010). In correlation with the findings of the current study, most of these studies showed improvement of probiotic survival due to encapsulation (Crittenden *et al.*, 2006; Ann *et al.*, 2007, Oliveira *et al.*, 2007b, Weinbreck *et al.*, 2010). However, O'Riordan *et al.* (2001) reported that encapsulation of a *Bifidobacterium* strain in starch did not improve its survival over time.

Though several studies done on shelf life have reported improved survival of probiotics due to encapsulation, most of them did not make comparison to unencapsulated cells (Oliviera *et al.*, 2000a; Hsiao *et al.*, 2004; Chavez and Ledeboer, 2007; Weinbreck *et al.*, 2010). In this study two controls were used. In the first one freeze-dried bacteria were physically mixed with the two polymers (PVP and PVAc-CA) to be used in the encapsulation process. In the second control, the polymers were reacted first before being mixed with unencapsulated bacteria. In both cases there was lower survival of *B. lactis* over the storage time compared to encapsulated bacteria. This showed that the polymers (processed or unprocessed) in themselves do not increase the shelf life of viable cells. Protection is only conferred when

these polymers are processed together with bacteria using the PGSS process to encapsulate the bacteria.

### 3.4.2 Water activity

Water activity is a crucial factor to consider in maintaining probiotic viability over the expected shelf-life for therapeutic benefits to be realised (Weinbreck *et al.*, 2010). An elevated water activity ( $>0.25$ ) negatively affects viability of microorganisms (Teixeira *et al.*, 1995; Weinbreck *et al.*, 2010). The effect of  $a_w$  was found to be the strongest factor in decreasing probiotic viability in a study done by Abe *et al.* (2009b). The best viability seems to be maintained by powders with a water activity of 0.2, which is equivalent to 4 % moisture content (Písecký *et al.*, 1997, Simpson *et al.*, 2005). A water activity of 0.56 was the most detrimental (Abe *et. al.*, 2009b).

In this study, an initial  $a_w$  of 0.34 was obtained for encapsulated bacteria while that of the controls was an average of 0.28. There was an increase in  $a_w$  of the samples between week 0 and week 3 (Table 3.1). Thereafter,  $a_w$  decreased for the duration of storage, culminating in an average of 0.27 for the controls and 0.33 for the encapsulated bacteria (Table 3.1). Low water activities of 0.24 – 0.37 obtained for all the tested samples for the duration of the storage period in this study were similar to those obtained by Crittenden *et al.* (2006). The highest water activity recorded for encapsulated bacteria microparticles in this study was 0.41, observed in the third week of storage. This level of water activity was accompanied by a high reduction in bacterial viability. However, though cell death was high, the levels of viable cells remained above the recommended minimum.

Table 3.1. Water activities of encapsulated and unencapsulated *B. lactis* Bb 12 samples stored in glass vials at 30 °C for 12 weeks.

Batch 1	Time (Weeks)					
	0	1	3	5	7	12
	Water activity measurement					
Encapsulated bacteria	0.34± 0.1	0.37± 0.3	0.41± 0.1	0.36± 0.02	0.30± 0	ND
Control 1(Unprocessed polymers + freeze-dried bacteria)	0.27± 0.06	0.32± 0.01	0.37± 0.02	0.31± 0.03	0.25± 0.01	ND
Control 2 (Processed polymers + freeze-dried bacteria)	0.28± 0.05	0.37± 0.02	0.39± 0.02	0.31± 0.04	0.24± 0	ND
Batch 2						
Encapsulated bacteria	0.34± 0.1	0.27± 0	0.41± 0.01	ND	0.33± 0	0.33±0
Control 1(Unprocessed polymers + freeze-dried bacteria)	0.27± 0.06	0.32± 0.01	0.28± 0.01	ND	0.27± 0.06	0.21±0. 03
Control 2 (Processed polymers + freeze-dried bacteria)	0.28± 0.05	0.37± 0.02	0.23± 0.01	ND	0.30± 0.06	0.33± 0.04

\*ND – not determined, Reported values are average of 2 samples ±standard deviation (SD)

Higher  $a_w$  values were recorded for the encapsulated sample as compared to the unencapsulated. However, higher mortality (reduction in number of viable cells) was obtained for the unencapsulated bacteria. This suggested that water activity was not the only factor causing a reduction in cell viability, but that other factors also contributed to bacterial cell death. The negative impact of these additional factors on encapsulated bacteria seems to have been reduced by the interpolymer complex matrix.

Water activities as low as 0.14 were obtained by Picot and Lacroix (2006) when they encapsulated bifidobacteria in whey protein-based microcapsules. With the exception of *Staphylococcus aureus* which can grow at water activities as low as 0.86, most pathogens and

other bacteria require a water activity above 0.94 (Cloete and Atlas, 2006). Therefore, spoilage microorganisms will not be able to grow in the encapsulated probiotic powder. This will help with regard to safety of the probiotic product.

### 3.4.3 Appearance of bacterial powder over time

Freshly encapsulated bacteria and unencapsulated bacteria are slightly yellow or cream in colour (Fig. 3.2A). After storage at 30 °C for a week discolouration becomes evident (Fig.3.2). Both encapsulated and unencapsulated cell powders became darker after some time in storage (Fig 3.2B). Unencapsulated bacterial powder (Fig. 3.2B, Y) showed a more intense colour compared to the encapsulated sample (Fig. 3.2B, X) upon visual inspection. Discolouration of dry powders in storage is unwanted and has been observed in foods such as milk powder and instant formula (Kurtmann *et al.*, 2009).



Fig 3.2 Encapsulated (X) and unencapsulated (Y) bacterial powder, (A) before storage and (B) showing browning after storage at 30 °C for 3 weeks.

Not only is discolouration undesirable, but it is also associated with loss in bacterial viability. In milk powders and infant formula the decrease in cell survival is believed to be as a result of the Maillard reaction (Guerra-Hernandez *et al.*, 2002; Thomsen *et al.*, 2005a, 2005b;

Kurtmann *et al.*, 2009). This is a non-enzymic reaction involving sugar-protein or sugar-peptide groups in foods, on heating or during prolonged storage (Fayle and Gerrard, 2002). It is accompanied by a loss in nutritional value, since the part of the protein that reacts with the sugar is not digested. Kurtmann *et al.* (2009) observed the loss of viability of freeze-dried probiotic bacteria during storage and suggested that cell death in storage could be caused by interplay of oxidation and browning reactions and stability of the bacterial cell wall. Water activity was found to play an important part role in these interactions. The results of this study indicate that encapsulation delayed the browning reaction, which could potentially be one of the mechanisms by which viability of the encapsulated probiotic was improved.

### 3.5 Conclusions

Probiotic survival is of great importance thus various means of ensuring this have been looked into, including encapsulation. Storage conditions should also be closely monitored. In this study high numbers of live bacteria were obtained at high temperatures (30 °C) after 12 weeks in storage. This result suggests the delivery of viable cells in sufficient numbers for the probiotic effect to be realised. Encapsulation seems to confer considerable protection to *B. lactis* Bb12, extending shelf-life by at least 7 weeks. There is need for the stability of this probiotic to be further tested in a dry product, which will take into account stresses experienced during technological processing.

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# Chapter 4

**Survival of poly-(vinylpyrrolidone)-poly-(vinylacetate-co-crotonic acid) (PVP: PVAc-CA) encapsulated *Bifidobacterium lactis* Bb12 in *mageu*, a traditional African maize-based fermented beverage.**

#### 4.1 Abstract

Traditionally, milk and other dairy products have been used as probiotic carriers. The major problem encountered with these products is that they are expensive, and are not accessible to some consumers, for example, poor rural consumers who cannot afford these products, and vegetarians. There is currently a need for incorporation of probiotic cultures into non-dairy products that will be relatively inexpensive and suitable for a wide range of consumers. In this study, the viability of a poly-(vinylpyrrolidone)-poly-(vinylacetate-co-crotonic acid) (PVP: PVAc-CA) interpolymer complex matrix encapsulated *Bifidobacterium lactis* Bb12 in *mageu*, a non-dairy maize-based fermented beverage, was investigated. *Mageu* was fermented, then pasteurised, before the probiotic strain was added. Viability of the probiotic strain and change in pH of *mageu* were monitored during the beverage's shelf-life under ambient conditions. The number of viable cells was analysed daily using a microplate scale fluorochrome assay with the LIVE/DEAD®*BacLight*™ kit and the plate count method. The pH of *mageu* with encapsulated probiotic remained almost constant over this period while that of *mageu* with unencapsulated probiotic decreased. The plate count method generally gave significantly lower viable counts than the microplate fluorochrome assay ( $p<0.05$ ). However, both methods showed levels of unencapsulated and encapsulated *B. lactis* Bb12 above the recommended therapeutic minimum ( $10^6$  cfu/ml) at the end of the beverage's shelf-life. The PVP: PVAc-CA matrix did not offer any significant protection to the *B. lactis* Bb 12 cells in the fermented product under the test conditions.

**Keywords:** Bifidobacteria, probiotic, LIVE/DEAD®*BacLight*™ kit, *mageu*, microplate fluorochrome assay, poly-(vinylpyrrolidone), poly-(vinylacetate-co-crotonic acid)

## 4.2 Introduction

There is an increasing interest in the use of probiotics as a natural means for enhancing health and combating disease due to growing resistance to commonly prescribed antibiotics (Levy, 2000; Kalui *et al.*, 2010). Probiotics are associated with several health benefits and are associated with lactic acid producing bacteria (Kalui *et al.*, 2010). Yoghurt and other fermented dairy products have conventionally been used as the most suitable delivery vehicle for probiotics. Dairy forms the bulk of the probiotic market, accounting for almost 33 % of the broad market (Granato *et al.*, 2010). However, due to milk-based carrier association with unfavourable cholesterol content and lactose intolerance as well as growing vegetarianism concerns, alternative probiotic carriers are being looked into. Probiotic yoghurts are also expensive and thus may not be consumed by the low income or rural populace (McMaster *et al.*, 2005). Currently, potential probiotic carriers include fruit-, vegetable-, meat-, soy-, and cereal-based products. These nondairy-based products contain reasonable amounts of carbohydrate, fibre, protein and vitamins (Yeo *et al.*, 2011). Cereals, in particular, are of interest because of their higher content of dietary fiber, increased amount of minerals such as phosphorous and higher content of essential vitamins compared to milk (Charalampopoulos *et al.*, 2002). Lactic acid bacteria perform an essential task in fermentation of cereals to preserve and produce wholesome foods.

Lactic acid fermentation of foods is widely practiced in African countries. Several fermented foods and beverages such as breads, pancakes, cheeses, milks, alcoholic and non-alcoholic drinks are available (Gadaga *et al.*, 1999). The fermented drinks include among others, *mahewu/mageu* (cereal-based), *amasi* (milk-based), sorghum or millet malt alcoholic beverages, and *makumbi* (fruit-based) (Gadaga *et al.*, 1999). The non-alcoholic beverage, *mageu*, is consumed in Africa and some Arabian Gulf countries (Chavan and Kadam, 1989; Blandino *et al.*, 2003). The drink is prepared from maize gruel and sorghum or millet malt or wheat flour is added and left at ambient temperatures for 24 hours to ferment (Odunfa *et al.*, 2001; Blandino *et al.*, 2003). The natural flora of the malt performs a spontaneous fermentation (Gadaga *et al.*, 1999). Sugar may be added to taste. The beverage is popular in summer as food and drink for labourers. It is also used as a weaning food (Simango, 1997; Gadaga *et al.*, 1999). These indicate that *mageu* is suitable for the whole family.

The predominant bacterium isolated from the fermentation of *mageu* in South Africa is *Lactococcus lactis* subsp. *lactis* (Steinkraus, 1996; Gadaga *et al.*, 1999). The beverage has

been shown to have bactericidal and/or bacteriostatic properties against strains of *Aeromonas*, *Salmonella*, *Shigella*, *Campylobacter jejuni* and enteropathogenic *Escherichia coli* (Simango and Rukure, 1991; 1992; Gadaga *et al.*, 1999). This suggests the beverage would not be a vehicle for transmission of pathogens.

Consumption of cereals and their products is more popular in rural areas because of their availability. *Mageu* can be used as a potential vehicle for probiotic delivery because it is cereal-based and widely-consumed. Addition of a probiotic strain into *mageu* would result in a functional food, which offers health benefits beyond its nutritional qualities (Champagne and Gardner, 2006). Health benefits can only be conferred to consumers provided sufficient amounts of probiotic are ingested. Probiotic products are normally kept under refrigerated conditions to ensure cells are viable throughout a product's shelf life. Refrigeration however, may not be common in rural areas where the beverage is mostly consumed. In this study a probiotic strain, *Bifidobacterium lactis* Bb12, encapsulated in an interpolymer complex matrix under supercritical CO<sub>2</sub>, was added to *mageu*. The aim was to determine whether the encapsulated probiotic would survive in sufficient amounts when the beverage was kept under ambient conditions, indicating the potential for use of *mageu* as a vehicle for delivery of probiotic cultures.

## 4.3 Materials and methods

### 4.3.1 Encapsulation of bifidobacteria

*Bifidobacterium lactis* Bb12 was obtained from Chr-Hansen as a freeze-dried preparation. Encapsulation was done according to Moolman *et al.* (2006) and Thantsha *et al.* (2009) without any modifications.

### 4.3.2 Preparation of *mageu*

Soft porridge was prepared by mixing 30 g of maize meal in 500 ml of water. The mixture was boiled for 10 min, cooled down, and then 30 g of sorghum malt added. The mixture was left to ferment spontaneously at room temperature for 24 h. The fermented *mageu* was

divided into 100 ml samples in Schott bottles and autoclaved, then each 100 ml aliquot aseptically inoculated with 1 g of either encapsulated or unencapsulated probiotic. Samples were then stored at room temperature for 5 days. Subsamples were taken over the storage period for viability analysis. Viability was measured using both the microtiter plate fluorochrome assay and plate counts.

#### 4.3.3 Measurement of pH

A Thermo Orion pH meter (model 410) was used to measure pH of 10 ml sub samples taken from the inoculated beverage daily over the 5-day period. Duplicate measurements were taken and the average reported.

#### 4.3.4 Pretreatment of *mageu* for analysis by microplate assay and plate counts

One milliliter of the *mageu* was suspended in 9 ml of sterile filtered (0.45 µm) simulated intestinal fluid (pH 6.8) (Section 3.3.3). The encapsulated bacteria were released by incubation of the suspension at 37 °C for 2 h and homogenized using a stomacher (Lab blender 400) for 30 min. Thereafter, the samples were centrifuged at 800 rpm for 7 min to remove large solid particles from the samples (Lahtinen *et al.*, 2006). The supernatant was further pelleted by centrifugation (5 min, 4000 rpm) and re-suspended in 0.85 % NaCl (Alakomi *et al.*, 2005). A Spectronic® 20 Genesys™ spectrophotometer was used to adjust the optical density of the solutions to 0.2 at 600 nm. The solutions were then analysed using plate count technique and microplate fluorochrome assay.

### 4.3.5 Microplate assay

#### 4.3.5.1 Preparation of standard curve

Twenty-five milliliter cultures of *Bifidobacterium lactis* Bb12 were grown in MRS broth supplemented with 0.05 % cysteine hydrochloride. The Erlenmeyer flasks (conical flasks) containing the cultures were incubated in anaerobic jars at 37 ° C for 72 h. Anaerocult® A (Merck, Pty, Ltd) was added for the production of anaerobic environment and Anaerotest® strips (Merck, Germany) included for indication of anaerobic conditions. The culture was concentrated by centrifugation at 10 000 g for 10-15 min. The supernatant was removed and the pellet re-suspended in 2 ml of 0.85 % NaCl. A milliliter of this suspension was added to each of two centrifuge tubes containing 20 ml of 0.85 % NaCl. One sample was heated for 5 min in a microwave set at high heat to kill the bacteria. Both samples were then centrifuged at 10 000 g for 10-15 min to pellet the cells, the supernatant removed and the centrifugation step repeated. Both pellets were re-suspended in separate tubes with 10 ml of 0.85 % NaCl. The optical density at 600 nm ( $OD_{600}$ ) was determined and adjusted to ~0.2 using a Spectronic® 20 Genesys™ spectrophotometer. Different volumes of live and dead bacteria were mixed to achieve different proportions of live: dead cells (0:100, 20:80, 50:50, 80:20 and 100:0) for determining a standard curve.

#### 4.3.5.2 Staining bacterial suspension

The fluorescent dyes used were propidium iodide (PI) and SYTO9 of the LIVE/DEAD® BacLight™ bacterial viability kit. Staining solution was prepared according to the manufacturer's instructions. The stock solutions dissolved in dimethyl sulfoxide were of the following concentrations: 3.34 mM for SYTO9 and 20 mM for PI. A mixture of 6 µl of both dyes was added to 2 ml of filter-sterilised dH<sub>2</sub>O to make a 2X staining solution (Molecular probes, invitrogen detection technologies).

#### 4.3.5.3 Microtiter plate fluorochrome assay

Fluorescence measurements in microplate scale were performed using the automated fluorometer Fluoroskan Ascent FL (Labsystems, Finland). Microtiter plate fluorochrome assay with LIVE/DEAD *BacLight* viability kit was performed as previously described by Alakomi *et al.* (2005), with minor modifications. Briefly, aliquots of 100 µl cell suspension (~1 x 10<sup>8</sup> cells ml<sup>-1</sup>) were pipetted in parallel into separate wells of a black 96-well fluorescence microplate in triplicate. Then 100 µl of the 2X staining solution were added and mixed thoroughly by pipetting up and down several times. The plate was then incubated in the dark for 15 min and placed in a fluorometer for measurement of fluorescence of bacterial suspensions. Green fluorescence was analysed using excitation at 485 nm and emission at 538 nm and red fluorescence excited at 530 nm and emitted at 630 nm. Ratios of intensities of green over red fluorescence (G/R ratio) were used to estimate the amount of colony forming units in the samples by using standard curves previously determined.

#### 4.3.6 Plate count technique

Samples of *mageu* were serially diluted 10 times in ¼ strength Ringer's solution. Aliquots of 0.1 ml were plated out in triplicate. The pour plate method was carried out using De Man, Rogosa and Sharpe (MRS) agar supplemented with 0.05 % cysteine hydrochloride. The plates were incubated in anaerobic jars at 37 °C for 72 h before enumeration. Anaerocult® A was added for the production of anaerobic environment and Anaerotest® strips (Merck, Germany) included for indication of anaerobic conditions.

#### 4.3.7 Statistical analysis

A one-way analysis of variance (ANOVA) was performed using StatSoft Statistica v10.

## 4.4 Results and discussion

### 4.4.1 Survival of encapsulated *B. lactis*

Probiotics have to be in sufficient amounts when they reach the colon to be of health benefit to the consumer. The therapeutic minimum has been stipulated at different levels. The minimum documented is  $10^6$ cfu/ml (Kurmann and Rasic, 1991; Bhadoria and Mahapatra1, 2011), while others suggest satisfactory levels of  $10^7$  and  $10^8$  cfu/ml or g (Donkor *et al.*, 2006).

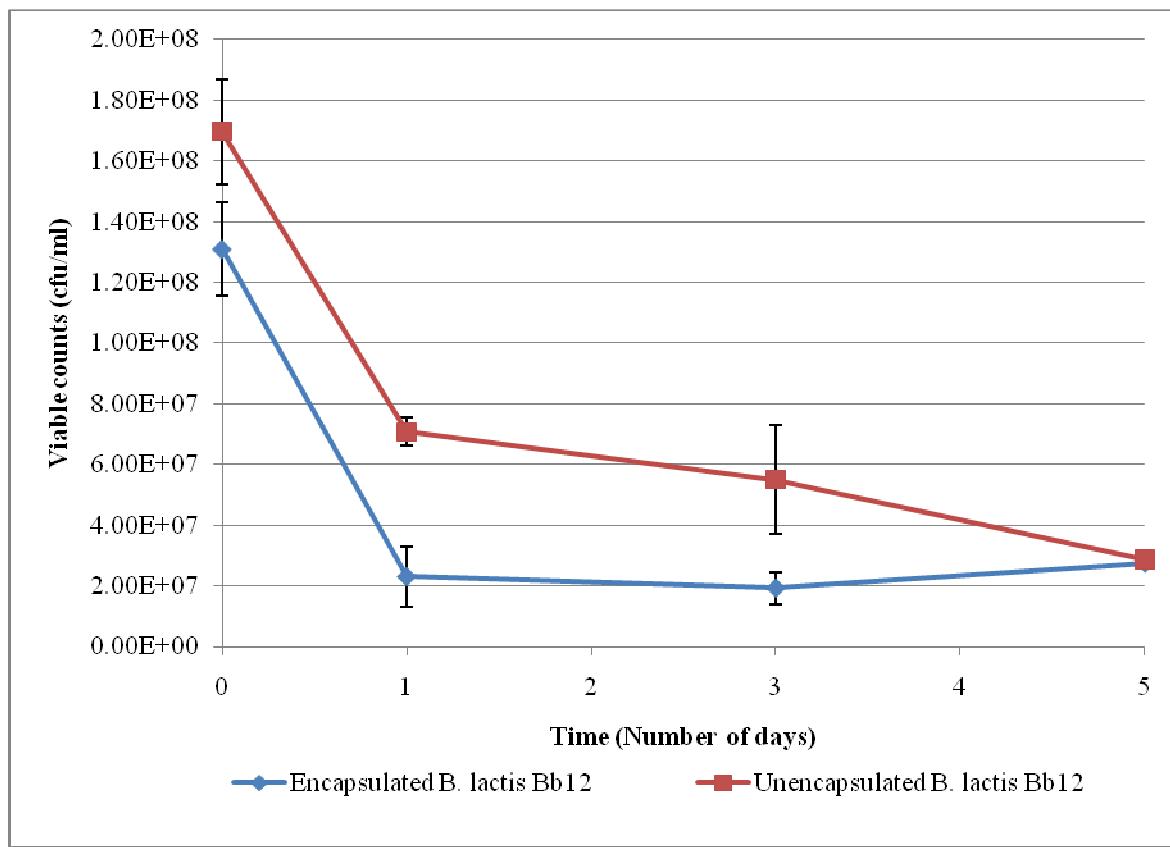


Fig. 4.1 Survival of encapsulated and unencapsulated *B. lactis* Bb 12 in mageu

In this study an initial amount of  $1.42 \times 10^8$ cfu/ml probiotic bacteria was released from the encapsulating matrix. After 5 days there were about  $2.7 \times 10^7$ cfu/ml (Fig 4.1), indicating a loss of only  $0.72 \log_{10}$  cfu/ml. In the same period the number of unencapsulated decreased from  $1.82 \times 10^8$ cfu/ml to  $3 \times 10^7$  cfu/ml ( $0.78 \log_{10}$  cfu/ml loss). In both encapsulated and unencapsulated samples, viable numbers decreased during the first day. Bifidobacteria are sensitive to low pH thus their numbers would decrease in such an environment. Although this would be expected for unencapsulated cells because of exposure to acids or low pH present in

*mageu*, the reason for the same decline in encapsulated cells could not be fully explained. Possibly, there may have been porous microparticles that allowed seepage of acidic *mageu* to the encapsulated cells, killing probiotics enclosed within. However, previous studies with the microparticles tested showed pH dependent swellability of the interpolymer complex matrix used in this study (Thantsha *et al.*, 2009), only occurring at pH values higher than those measured in *mageu*.

There was a decline in the number of viable cells in the unencapsulated sample from day 3, from  $6.76 \times 10^7$  cfu/ml to  $3.09 \times 10^7$  cfu/ml on day 5. However, encapsulated bacteria remained almost constant after the initial decline over the test period (Fig 4.1). Encapsulation seems to offer some protection against acidity during this time. The advantage of number of viable cells remaining almost constant is that the amount of metabolites produced by *B. lactis* that are likely to alter the taste of the beverage, would be minimal. It was noted that survival of encapsulated bacteria did not differ significantly from that of unencapsulated bacteria ( $p>0.05$ ). Encapsulation should ideally create a micro-environment where probiotic cells are protected from high acid. Microencapsulation was shown to significantly protect probiotics from low pH (3.5) in a study by McMaster *et al.* (2005). They used *mageu* as a vehicle to deliver gellan/xanthan-encapsulated *B. lactis* DSM 10140.

*Mageu* has a relatively short shelf life under non-refrigerated conditions (1-2 days) (Simango, 1997). The beverage in this study was monitored for 5 days. Both samples contained viable bacteria above the recommended minimum at the end of the test. Thus there will be a sufficient number of *B. lactis* Bb12 at the end of its shelf-life, even when kept at ambient temperatures.

#### 4.4.2 Change in pH

Fermentation results in the reduction of pH. A low pH discourages the growth of pathogens, thus increasing shelf-life of fermented foods. However if increase in acidity is unlimited it might negatively affect acceptability of the product by the consumer due to bitterness. Therefore there is need to achieve a balance of pH in a product.

*B. lactis* Bb12 was added after fermentation and pasteurisation, ensuring that live numbers only came from the added probiotic. The initial pH in the encapsulated probiotics sample was

4.65 (Table 4.1). At the end of 5 days the final pH was an average of 4.40, indicating a decrease of only 0.20. Since the change in pH of the sample (for example from pH 4 to pH 3) represents a 10 fold change in acidity, the change in pH of the sample where encapsulated probiotic was added can be described as insignificant (Hodges, 2003).

Table 4.1 pH values of *mageu* samples with encapsulated and unencapsulated *B. lactis* Bb12

Culture	Day					
	0	1	2	3	4	5
Encapsulated	4.65±0.06	4.63±0.11	4.60±0.08	4.54±0.04	4.50±0.06	4.40±0.06
Unencapsulated	4.94±0.11	4.90±0.35	4.87±0.26	4.80±0.19	4.70±0.06	4.53±0.15

n=9 for encapsulated probiotic; n=3 for unencapsulated probiotic. Values given ± SD

For the unencapsulated probiotic sample a slightly higher pH drop was observed, from an initial of 4.94 to 4.53. This pH drop was almost double that noted in the encapsulated *B. lactis* Bb12 sample. This would suggest that encapsulation protects the product from changes in pH. Any metabolites that are produced by cells are kept inside the matrix, and are not released into the product thereby altering the pH.

Microencapsulation should ensure targeted release of any bioactive. Release of encapsulated probiotics from the encapsulating PVP: PVAc-CA matrix has been shown to be pH-dependent (Thantsha *et al.*, 2009). The matrix remains intact at low pH (pH 2), releasing the encapsulated bacteria at pH 6.8. The pH at which the matrix swells/disintegrates to release encapsulated bioactive corresponds to that found in the colon, which is near neutral. It would therefore be expected that at the pH of fermented *mageu* (pH 4 -5) encapsulated *B. lactis* cells would not be released from the encapsulating matrix, and thus be protected from the acidity. However, the decline in viable numbers over the first day would suggest that cells encapsulated were affected by the acid in *mageu* (Fig. 4.1). A possible reason for this could be due to the porous surface observed on some microcapsules as described earlier (Mamvura *et al.*, 2011). Although the pores were small enough to retain cells, some beverage may have seeped in. The decrease in viable numbers

was, however, not significant. Liserre *et al.* (2007) noticed the same unexpected problem on alginate-chitosan beads encapsulating *B. animalis* subsp. *lactis* at pH 4.5. They highlighted the need to test stability of microcapsules over a pH range to ensure potential maximal protection in fermented food products.

*Mageu* in this study reached a final pH of 4.53 and high viable numbers ( $3.09 \times 10^7$  cfu/ml) were still obtained, even for unencapsulated bacteria. In a different study, unencapsulated *B. lactis* remained stable in an oat-based fermented product (pH below 4.5) over 25 days (Lahtinen *et al.*, 2005). This could be because *B. lactis* Bb12 has a high resistance to low pH (Vernazza *et al.*, 2006). In their study Vernazza *et al.*, (2006) showed that there was increased survival of this strain as compared to *B. longum*, *B. adolescentis* and *B. infantis* strains tested. Survival was higher at pH 4 than at pH 2. Tolerance of *B. lactis* to low pH is believed to be caused by induction of H<sup>+</sup>-ATPase activity (Matto *et al.*, 2006). H<sup>+</sup>-ATPase is the enzyme responsible for maintaining pH homeostasis in bacterial cells when exposed to acidic conditions. When this enzyme's activity is induced H<sup>+</sup> protons are discharged for maintenance of a constant intracellular pH (Matsumoto *et al.*, 2004).

#### 4.4.3 Comparison of enumeration methods

The viability of probiotics has been traditionally assessed with the plate count method. This method has been criticized for being laborious and time consuming. A rapid method that utilizes fluorescent nucleic acid stains in the LIVE/DEAD® *BacLight*™ kit in a microplate fluorescent reader was developed (Alakomi *et al.*, 2005). The kit contains SYTO9 which stains both live and dead cells green. When used together with propidium iodide (PI), SYTO9 is displaced from cells with damaged membranes and PI is taken up. The viability stains distinguish live (green-fluorescent) from dead (red-fluorescent) cells. However, the stains on their own are qualitative, only distinguishing between live, damaged and dead bacteria. For enumeration of the stained cells, the stains are analysed using either microscopy or fluorometry.

The traditional plate counting technique was compared with the microplate fluorochrome assay as enumeration techniques. A standard curve was constructed for reference for the fluorometry method. Single culture powdery probiotic preparations typically contain  $10^{10}$

to  $10^{11}$  cfu/g (Saarela *et al.*, 2000). A linear relationship between relative fluorescence units and viable counts was found to be around log 6-7 cfu/ml (Alakomi *et al.*, 2005). There was therefore a need for samples to be diluted before assessment. Furthermore, *mageu* has a cloudy appearance and this would interfere with absorbance measurements in the preparation of samples before the fluorometric assay. A ten-fold dilution was made. Calculations of viable counts were taken from the standard curve and the dilution factor taken into account.

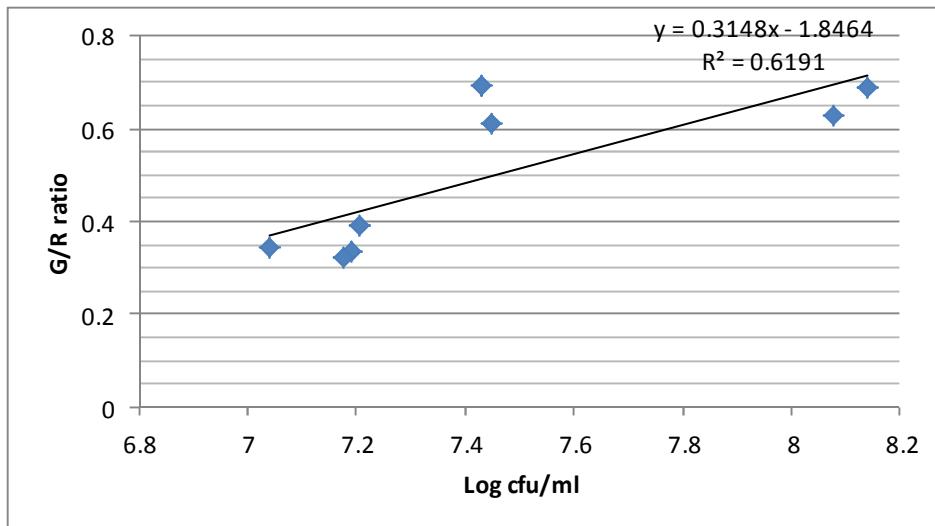


Fig. 4.2 Correlation between relative viability (fluorometry) and plate counts.  $R^2=0.62$

The results on the relative viability of *B. lactis* Bb12 in *mageu* correlated poorly ( $R^2=0.62$ ) with plate counts (Fig. 4.2). The fluorometry results differed significantly from plate count results ( $p<0.01$ ). This result was in agreement with the findings of Lahtinen and colleagues (2006). The plate count method gave lower counts than those obtained with the microplate assay (Table 4.2). However, Alakomi *et al.* (2005) found that there was a good correlation between the microplate fluorochrome assay and plate count results.

Table 4.2 The changes in numbers of *B. lactis* Bb12 in *mageu* stored at room temperature for 5 days enumerated with plate counts and microplate fluorochrome assay

Sample	Time (days)	Plate count ( $\log_{10}$ cfu/ml)	Fluorochrome assay ( $\log_{10}$ cfu/ml)
Encapsulated	0	8.16±0.08	8.41±0.08
	1	7.49±0.51	8.12±0.06
	3	7.37±0.44	8.10±0.06
	5	7.43±0.01	8.33±0.2
Unencapsulated	0	8.26±0.5	8.38±0.08
	1	7.87±0.08	8.24±0.1
	3	7.83±0.06	8.17±0.01
	5	7.49±0.1	8.12±0.03

Reported values are average ± standard deviation (SD), n = 3

Underestimation of viable numbers by the plate count method may be due to the state of bacteria. There may be live cells which cannot be cultured, often described as ‘dormant’ bacteria (Kell *et al.*, 1998; Lahtinen *et al.*, 2006). Such cells may be unable to grow on conventional media but stained appropriately by viability stains. Lahtinen *et al* (2005) suggest that plate count techniques and the use of live/dead kits measure different subpopulations of bacteria hence the difference in results. Counts obtained from viability kits show readily culturable cells and those with intact membranes, which might not form colonies when grown on conventional media.

## 4.5 Conclusions

Traditional fermented foods offer a cheap and affordable means to act as probiotic vehicles. Survival of *B. lactis* in *mageu* has been shown to be feasible in this study. At ambient temperatures there were still viable counts above the recommended therapeutic minimum at the end of the beverage’s shelf life. However, encapsulation did not seem to offer significant protection to cells. It prevented a rapid decrease of pH in *mageu*, suggesting that any metabolites produced by cells are kept within the microcapsules. Thus, the taste (sourness or bitterness due to acidity) of *mageu* may not be altered negatively by addition of encapsulated probiotic *B. lactis* Bb12.

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# Chapter 5

## General Conclusions and Recommendations

## Conclusions

- The PVP: PVAc-CA microparticles encapsulating probiotic *B. lactis* Bb12, prepared using the PGSS process with supercritical CO<sub>2</sub> as solvent, were small, with their size falling within an acceptable size for microparticles for application in food products.
- These microparticles were approximately uniform in shape, with most of them spherical and others irregular. The spherical shape of the microparticles suggesting will promote their even distribution when incorporated into foods.
- The microparticles had high encapsulation efficiency, with the bacteria evenly distributed within the microparticles, as depicted by CSLM. No bacterial cells were observed on the surfaces of microparticles, indicating that all the bacteria were encapsulated. This will ensure that probiotics encapsulated using this method will be delivered live in high quantities.
- High temperatures are detrimental to cells. Encapsulation of *B. lactis* Bb12 within the PVP: PVAc-CA microparticles prolonged the shelf life (viable counts above recommended therapeutic minimum of  $1 \times 10^6$  cfu/ml) of these bacterial cells at 30°C by five weeks, extending it from 7 weeks when unencapsulated to 12 weeks when encapsulated.
- Water activities of encapsulated probiotic powder were within the acceptable range for dry products. The water activites of the encapsulated products were at levels unsuitable for growth of most pathogens, thus suggesting that the chances for pathogen growth and proliferation will be minimal, thus ensuring a safe product for consumption.
- Encapsulation seemed to slow down discolouration in the probiotic powder. Discolouration is associated with browning reactions and loss of bacterial viability. Thus one of the mechanisms by which encapsulation in the interpolymer complex improved viability of the probiotic cells may be through delaying of the Maillard reaction.

- Encapsulation did not seem to offer significant protection to *B. lactis* Bb12 cells when incorporated into a traditional fermented beverage, *mageu*. However, pH decrease was less in the product where encapsulated probiotic was added. This indicated that the cells within the microparticles were inactive in *mageu* or that if they are active, any metabolites produced by encapsulated *B. lactis* Bb 12 seemed to be kept within the microparticles and not released into *mageu*, thus ensuring that taste will not be altered.
- The presence of high levels of *B. lactis* Bb 12 obtained at the end of *mageu*'s shelf-life under ambient conditions suggests that this beverage can be used as a vehicle for delivery of *B. lactis* Bb12 probiotic cells. However, the results indicated that there will be no difference in viability between encapsulated and unencapsulated *B. lactis* Bb12 cells incorporated into *mageu*.

### **Recommendations for future work**

- Shelf-life stability of encapsulated probiotics needs to be checked over temperature variations to simulate conditions encountered during transportation and storage. A constant temperature may not be indicative of the situation in the food industry.
- There is need for release of probiotic from the encapsulating material to be tested over a pH range to determine when disintegration of the matrix starts to occur. Problems occur in fermented products when protection from the high acid may not be fully achieved.
- Strains which are more susceptible to high acid need to be incorporated into fermented foods to test for their survival. *B. lactis* is known for its high acid tolerance and thus may not show effect of acid on live cells. However, for application in acidic foods this strain is still highly recommended for its hardiness.