# CHAPTER 3

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

**Published:** F. S. Moolman, P. W. Labuschagne, M.S. Thantsha, T. L. van der Merwe, H. Rolfes and T. E. Cloete. (2006). Encapsulating probiotics with an interpolymer complex in supercritical carbon dioxide. South African Journal of Science 102, 349-354.

## 3.1 ABSTRACT

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

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

## 3.2 INTRODUCTION

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

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

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

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

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

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

The challenges in developing commercially viable encapsulated products depend on:

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

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

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

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

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

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

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

| Approach | Elaboration | Limitations | Reference |
|---|---|---|---|
| Changing polymer design | Incorporation of "$CO_2$-philic" functional groups in new polymers | FDA approval needed for new polymers | Sarbu et al., 2000 |
| Surfactants | Addition of $CO_2$ soluble surfactants | FDA approval needed for surfactants | Hoefling et al., 1993; McClain et al., 1996; Yazdi et al., 1996 |
| Cosolvents | Addition of cosolvents , e.g. methanol or ethanol, to increase the solvation power of $scCO_2$ | Reintroduces requirement for use of a solvent-many actives are sensitive to solvents | Ekart et al., 1993; Kazarian et al., 1998; Mishima et al., 2001; Corrigan and Crean, 2002 |
| Mixtures of SCFs | The use of $2^{nd}$ SCF to enhance polymer processability | No obvious $2^{nd}$ SCF with desired combination of properties (low/no toxicity, low critical temperature and pressure, low cost, etc.) | |
| Gas anti-solvent technique | Use of $scCO_2$ as an anti-solvent to extract the solvent from a sprayed polymer solution and thus precipitate the polymer | Reintroduces requirement for use of a solvent-many actives are sensitive to solvents | Subramanian et al., 1999 |
| Use of low molar mass and low polarity polymers | Polymers are more amenable to $scCO_2$ processing | These polymers generally have low mechanical integrity and/or barrier properties | Rindfleisch et al., 1996 |
| Use of fats/waxes for encapsulation | Fats, waxes and oils are generally soluble in $scCO_2$ | Limited flexibility with regards to properties | Benoit et al., 2000 |

## 3.3 MATERIALS AND METHODS

### 3.3.1 Bacterial cultures

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

### 3.3.2 Encapsulation of bacteria

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


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

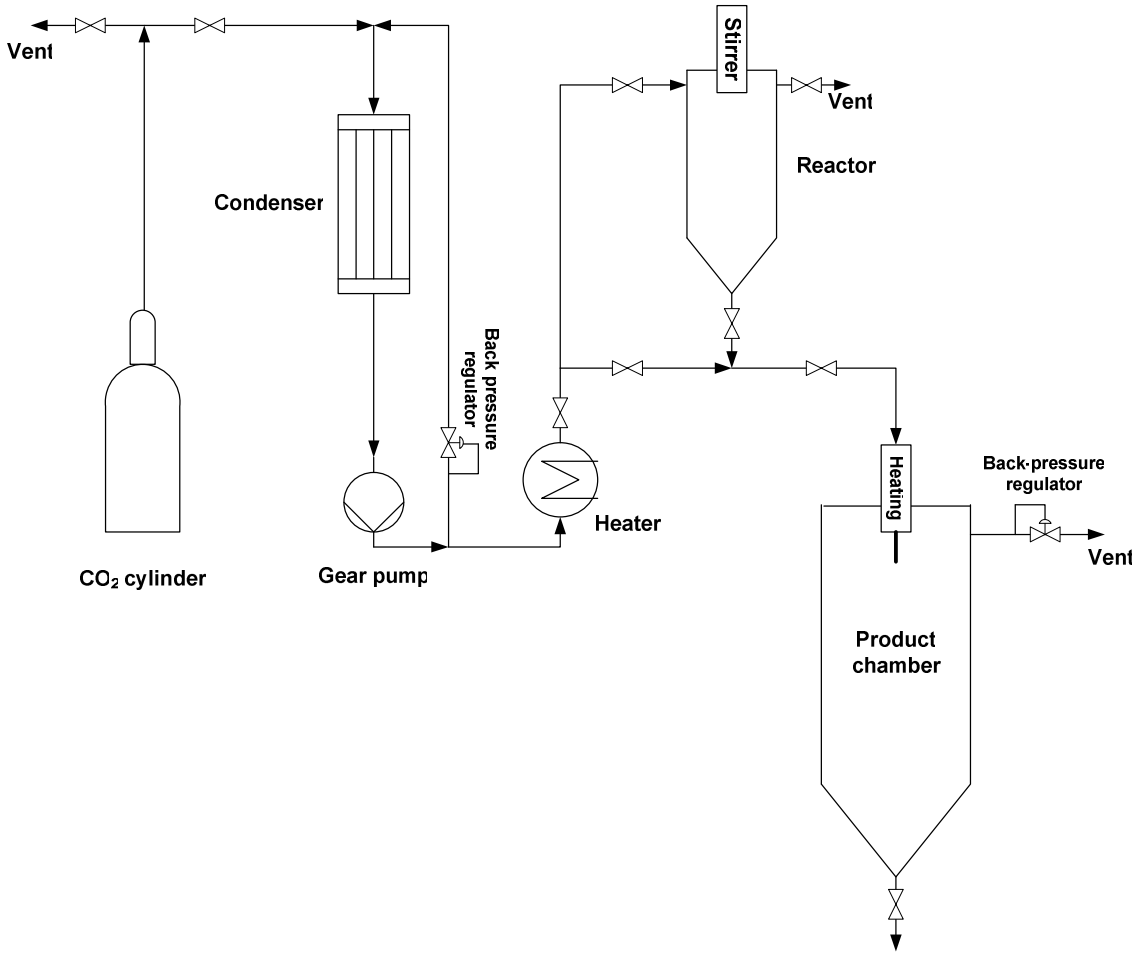


**Figure 3.2: A simplified process flow diagram of the PGSS (Particles from Gas-Saturated Solution) system used to produce encapsulated probiotics**

### 3.3.3 Scanning electron microscopy (SEM)

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

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

### 3.3.4 Bacterial counts

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

## 3.4 RESULTS AND DISCUSSION

### 3.4.1 Liquefaction of polymers in $scCO_2$

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

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

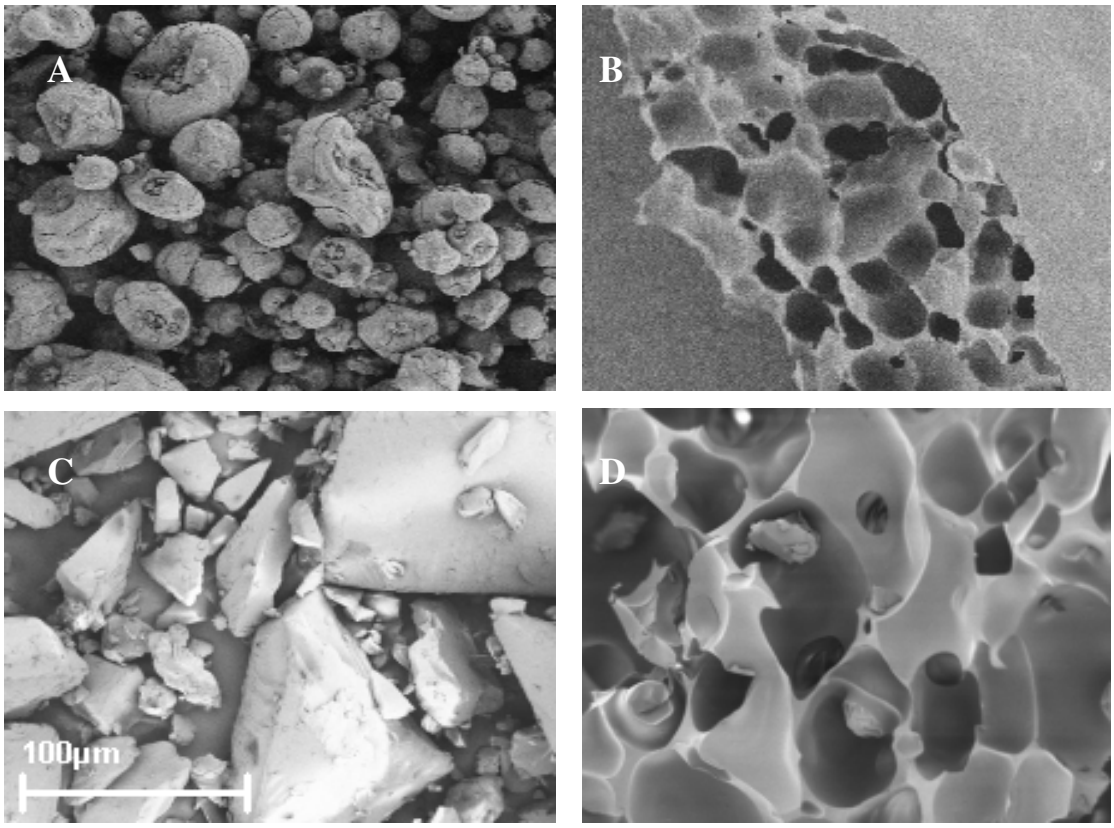


**Figure 3.3: Comparison of PVP before (A) and after (B) and VA-CA before (C) and after (D) $scCO_2$ exposure**

### 3.4.2. Interpolymer complexation and bacterial encapsulation

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

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

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

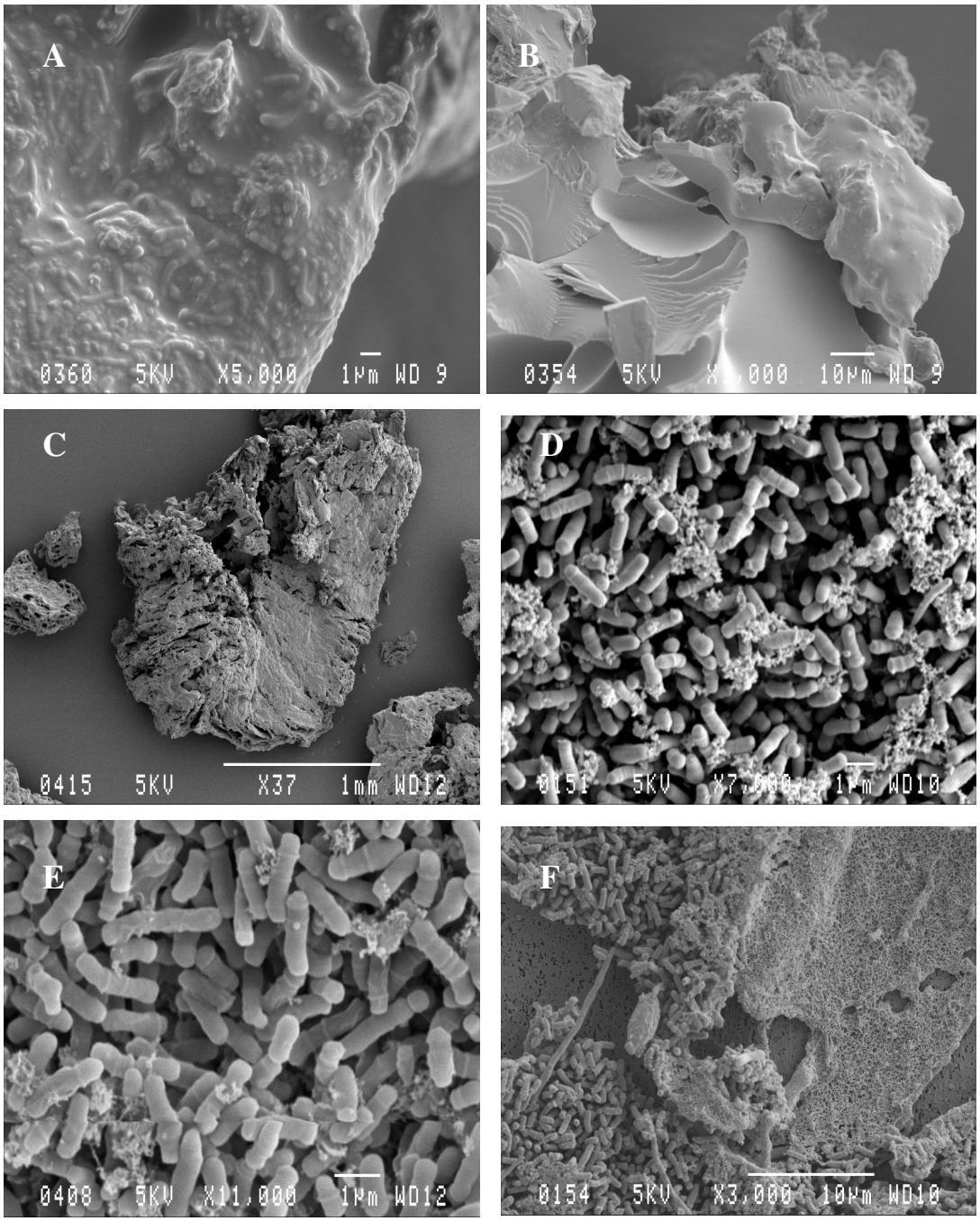


**Figure 3.4: SEM images of *Bifidobacterium longum* Bb-46 cells: A and C: Non-encapsulated (powder), B: scCO$_2$ interpolymer complex encapsulated (powder), D: Non-encapsulated (suspension), E and F: scCO$_2$ interpolymer complex encapsulated (suspension)**

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

### 3.4.3 Appearance of non-encapsulated and encapsulated cells upon suspension

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

[1] Moolman F. S. Polymers, Ceramics and Composites, Materials Science and Manufacturing, CSIR, P. O. Box 395, Pretoria, 0001, South Africa

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

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

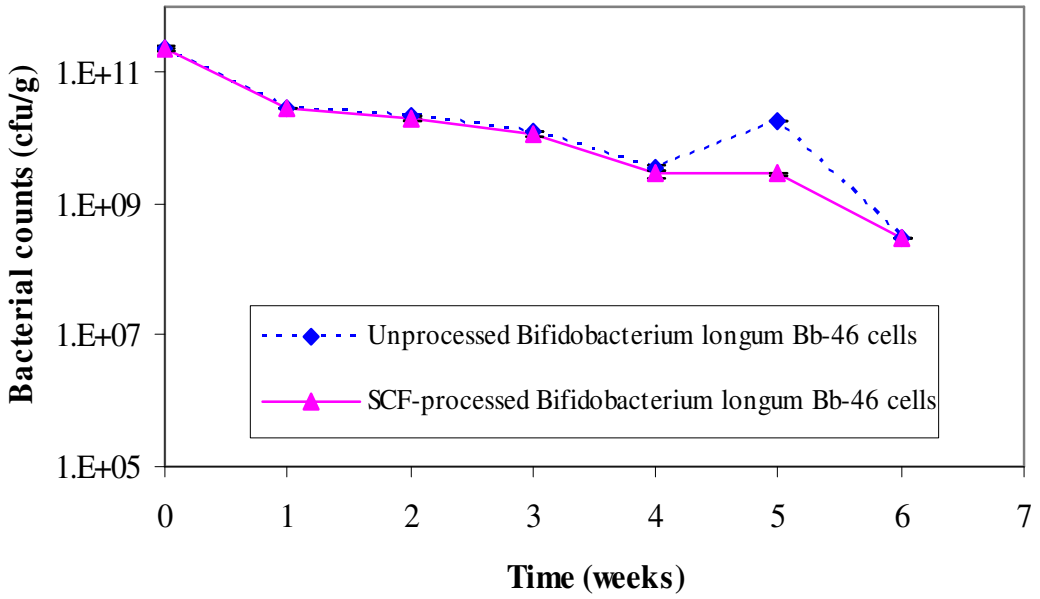


**Figure 3.5: Survival of freeze-dried bacteria and freeze-dried bacteria that went through the encapsulation process during storage**

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

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

## 3.5 CONCLUSIONS

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

## 3.6 REFERENCES

Bae, W., Kwon, S., Byun, H-S., Kim, H., 2004. Phase behavior of the poly (vinyl pyrrolidone) + N-vinyl-2-pyrollidone + carbon dioxide system. Journal of Supercritical Fluids 30, 127-137.

Benoit, J.P., Rolland, H., Thies, C., Vande Velde, V., 2000. Method of coating particles and coated spherical particles. United States of America Patent no. 6,087,003

Bielecka, M., Biedrzycka, E., Majkowska, A., 2002. Selection of probiotics and prebiotics for synbiotics and confirmation of their in vivo effectiveness. Food Research International 35, 125-131.

Corrigan, O.I., Crean, A.M., 2002. Comparative physiochemical properties of hydrocortisone-PVP composites prepared using supercritical carbon dioxide by the GAS

anti-solvent recrystallization process, by coprecipitation and by spray drying. International Journal of Pharmaceutics 245, 75-82.

Demirbaş, A., 2001. Supercritical fluid extraction and chemicals from biomass with supercritical fluids. Energy Conversion and Management 42, 279-294.

Doleyres, Y., Lacroix, C., 2005. Technologies with free and immobilized cells for probiotic bifidobacteria production and protection. International Dairy Journal 15, 973-988.

Ekart, J., Bennet, K.L., Ekart, S.M., Gurdial, G.S., Liotta, C.L., Eckert, C.A., 1993. Cosolvent Interactions in Supercritical Fluid Solutions. AIChE Journal 39, 235-248.

Elliot E., Teversham, K., 2004. An evaluation of nine probiotics available in South Africa, August 2003. South African Medical Journal 94, 121-124.

Fages, J., Lochard, H., Letourneau, J-J., Sauceau, M., Rodier, E., 2004. Particle generation for pharmaceutical applications using supercritical fluid technology. Powder Technology 141, 219-226.

Ginty, P.J., Whitaker, M.J., Shakesheff K.M., Howdle, S.M., 2005. Drug delivery goes supercritical. Materials Today 8, 42-48.

Havenaar, R., Ten Brink, B., Huis in 't Veld, J.H.J., 1992. Selection of strains for probiotic use. In: Fuller, R. (eds). Probiotics, the Scientific Basis. Chapmann & Hall, London., pp. 209-224.

Henke, A., Kadlubowski, S., Ulanski, P., Rosiak, J.M., Arndt K-F., 2005. Radiation-induced cross-linking of polyvinylpyrrolidone-poly (acrylic acid) complexes. Nuclear Instruments and Methods in Physics Research B 236, 391-398.

Hoefling, T.A., Beitle, R.R., Enick, R.M., Beckman, E.J., 1993. Design and Synthesis of Highly $CO_2$ Soluble Surfactants and Chelating Agents. Fluid Phase Equilibria 82, 203-212.

Holzapfel W.H., Haberer, P., Snel, J., Schillinger, U., Huis in't Veld, J.H.J., 1998. Overview of gut flora and probiotics. International Journal of Food Microbiology 41, 85-101.

Huff, B., 2004. "Probiotics" may not be what they seem. Canadian Family Physician 50, 583-587.

Jung, J., Perrut, M., 2000. Particle design using supercritical fluid: literature and patent survey. Journal of Supercritical fluids 20, 179-219.

Kazarian, S.G., Vincent, M.F., West, B.L., Eckert, C.A., 1998. Partitioning of Solutes and Cosolvents between Supercritical $CO_2$ and Polymer Phases. Journal of Supercritical Fluids 13, 107-112.

Kirby, C.F. McHugh, M.A., 1999. Phase Behavior of Polymers in Supercritical Fluid Solvents. Chemical Reviews 99, 565-602.

Krasaekoopt, W., Bhandari, B., Deeth, H., 2003. Evaluation of encapsulation techniques of probiotics for yoghurt. International Dairy Journal 13, 3-13.

Kumar, V., Yang, T., Yang, Y., 1999. Interpolymer complexation. I. Preparation and characterization of a polyvinyl acetate phthalate-polyvinylpyrrolidone (PVAP-PVP) complex. International Journal of Pharmaceutics 188, 221-232.

Leahy, S.C., Higgins, D.G, Fitzgerald, G.F, van Sinderen, D., 2005. Getting better with bifidobacteria. Journal of Applied Microbiology 98, 1303-1315.

MacFarlane, G.T., Cummings, J.H., 1999. Probiotics and prebiotics: can regulating the activities of the intestinal bacteria benefit health? British Medical Journal 318, 999-1003.

Matsumoto M., Mochiduki, K., Kondo, K., 2004. Toxicity of ionic liquids and organic solvents to lactic acid-producing bacteria. Journal of Bioscience and Bioengineering 98, 344-347.

McClain, J.B., Betts, D.E., Canelas, D.A., Samulski, E.T., DeSimone, J.M., Londono, J.D., Cochran, H.D., Wignall, G.D., Chillura-Martino, D., Triolo, R., 1996. Design of Nonionic Surfactants for Supercritical Carbon Dioxide. Science 274, 2049-2052.

Micanel, N, Haynes, I.N., Playne, M.J., 1997. Viability of probiotics cultures in commercial Australian yoghurts. Australian Journal of Dairy Technology 52, 24-26.

Miguel, C.B, 2001. Microbiota and the gastrointestinal system. Revista Espanola de Enfermedades Digestiva 93, 328-330.

Mishima, K., Matsuyama, K., Hayashi, K., Ishikawa, H., Hirabaru, T., 2001. Formation of Polymer Microcapsules by Rapid Expansion of Supercritical Solution with a Nonsolvent. IUPAC Chemrawn XIV Conference

Moolman, F.S., Rolfes, H., Van der Merwe, T.L., 2005. Method of encapsulating an active substance. United States of America Patent Application no. 20050112205.

Moolman, F.S., Labuschagne, P.W., M.S., Thantsha, van der Merwe, T.L., Rolfes, H., Cloete, T.E., 2006. Encapsulating probiotics with an interpolymer complex in supercritical carbon dioxide. South African Journal of Science 102, 349-354.

Novik, G., Gamian, A., Fransisco, J., Dey, E.S., 2006. A novel procedure for the isolation of glycolipids from *Bifidobacterium adolescentis* 94 BIM using supercritical carbon dioxide. Journal of Biotechnology 121, 555-562.

O'Neill, M.L., Cao, Q., Fang, M., Johnston, K.P., 1998. Solubility of Homopolymers and Copolymers in Carbon Dioxide. Industrial & Engineering Chemistry Research 37, 3067-3079.

Picot, A., Lacroix, C., 2004. Encapsulation of bifidobacteria in whey protein-based microcapsules and survival in simulated gastrointestinal conditions and in yoghurt. International Dairy Journal 14, 505-515.

Raveendran, P., Ikushima, Y., Wallen, S.L., 2005. Polar Attributes of Supercritical Carbon Dioxide. Accounts of Chemical Research 38, 478-485.

Reverchon, E., Porta, G.D., 2001. Supercritical fluids-assisted micronization techniques. Low-impact routes for particle production. Pure and Applied Chemistry 73, 1293-1297.

Rindfleisch, F., DiNoia, T.P., McHugh, M.A., 1996. Solubility of Polymers and Copolymers in Supercritical $CO_2$. Journal of Physical Chemistry 100, 15581-15587.

Rolfes, H., Van der Merwe, T.L., Truter, P.-A. 2001. Method of making controlled release particles of complexed polymers. United States of America Patent no. 6,221,399

Salminen, S., Isolauri, E., Salminen, E., 1996. Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains and future challenges. Antonie van Leeuwenhoek 70, 347-358.

Salminen, S., Bouley, C.H., Boutro-Ruault, M.C., Cummings, J.H., Franck, A., Gibson, G.R., Isolauri, E., Moreau, M.C., Roberfroid, M.B., Rowland, I., 1998. Functional food science and gastrointestinal physiology and function. British Journal of Nutrition 80, S147-S171.

Sarbu, T., Styranec, T., Beckman, E.J., 2000. Non-fluorous polymers with very high solubility in supercritical $CO_2$ down to low pressures. Nature 405, 165-167.

Sardessai, Y., Bhosle, S., 2002. Tolerance of bacteria to organic solvents. Research in Microbiology 153, 263-268.

Sarrade, S., Guizard, C., Rios, G.M., 2003. New applications of supercritical fluids and supercritical fluid processes in separation. Separation and Purification Technology 32, 57-63.

Sihvonen, M., Järvenpää, E., Hietaniemi, V., Huopalahti, R., 1999. Advances in supercritical carbon dioxide technologies. Trends in Food Science and Technology 10, 217-222.

Siuta-Cruse, P. Goulet, J., 2001. Improving probiotic survival rates. Food Technology 55, 36-42.

Stormo, K.E., Crawford, R.L., 1992. Preparation of encapsulated microbial cells for environmental applications. Applied and Environmental Microbiology 58, 727-730.

Subramaniam, B., Saim, S., Rajewski, R. A., Stella, V., 1999. Methods for particle micronization and nanonization by recrystallization from organic solutions sprayed into a compressed antisolvent. United States of America Patent no. 5,874,029.

Talwalkar, A., Kailaspathy, K., Peirs, P., Aramugaswamy, R., 2004. Application RBGR-a simple way for screening of oxygen tolerance in probiotic bacteria. International Journal of Food Microbiology 71, 245-248.

Teitelbaum, J.E., Walker, W.A., 2002. Nutritional impact of pre- and probiotics as protective gastrointestinal organisms. Annual Review of Nutrition 22, 107-138.

Tilly, K.D., Foster, N.R., Macnaughton, S.J., Tomasko, D.L., 1994. Viscosity Correlations for Binary Supercritical Fluid Mixtures. Industrial & Engineering Chemistry Research 33, 681-688.

Tsuchida, E., 1994. Formation of polyelectrolyte complexes and their structures. Pure and Applied Chemistry A31, 1-15.

Tuohy, K.M., Probert, H.M., Smejkal, C.W., Gibson, G.R., 2003. Using probiotics and prebiotics to improve gut health. Drug Discovery Today 8, 692-700.

Vasistha, N., 2003. Microencapsulation: Delivering a market advantage. http://www.preparedfoods.com

Vinderola, C.G., Bailo, N., Reinheimer, J.A., 2000. Survival of probiotic microflora in Argentinian yoghurts during refrigerated storage. Food Research International 33, 97-102.

Yazdi, A.V., Lepilleur, C., Singley, E.J., Liu, W., Adamsky, F.A., Enick, R.M., Beckman, E.J., 1996. Highly Carbon Dioxide Soluble Surfactants, Dispersants and Chelating Agents. Fluid Phase Equilibria 117, 297-303.

Yeo, S.D., Kiran, E., 2005. Formation of polymer particles with supercritical fluids: A review. Journal of Supercritical Fluids 34, 287-308.

Yue, B., Yang, J., Wang, Y., Huang, C-Y., Dave, R., Pfeffer, R., 2004. Particle encapsulation with polymers via *in situ* polymerization in supercritical $CO_2$. Powder Technology 146, 32-45.