

CHAPTER 6

**Shelf life studies of *Bifidobacterium*
longum Bb-46 encapsulated in
interpolymer complexes in supercritical
CO₂ stored under different storage
conditions**

6.1 ABSTRACT

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

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

6.2 INTRODUCTION

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

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

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

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

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

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

6.3 MATERIALS AND METHODS

6.3.1 Encapsulation of bacteria

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

6.3.2 Pressing of tablets

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

6.3.3 Storage of samples

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

6.3.4 Enumeration of bacteria

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

6.3.5 Staining of bacteria

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

6.3.6 Confocal Laser Scanning Microscopy (CLSM)

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

6.3.7 Water activity (a_w) measurement

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

6.4 RESULTS AND DISCUSSION

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

The encapsulated bacteria were harvested from the product chamber and incubated in Ringer's solution (pH 6.8) for 2 h for release of encapsulated bacteria, unless specified otherwise. Throughout the storage time of 5 weeks at 30 °C, viable counts of non-encapsulated bacteria remained above the recommended effective minimum for probiotics. On the other hand, counts of viable bacteria released from the interpolymer matrix processed at 0 bar expansion pressure in the product chamber, were at the levels above 10⁸ cfu/g after 2 weeks of storage but were undetectable from 3 weeks onwards (Fig. 6.1). These results indicated the possibility of death of encapsulated cells or alternatively insufficient release of the cells from the interpolymer matrix due to the release media used.

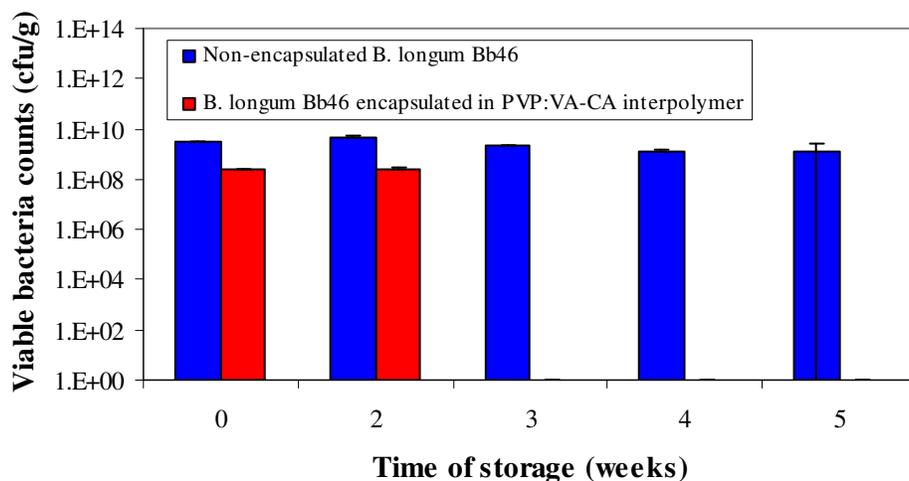


Figure 6.1: Survival of PVP:VA-CA encapsulated *B. longum* Bb-46 processed at 0 bar expansion pressure in the product chamber

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

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

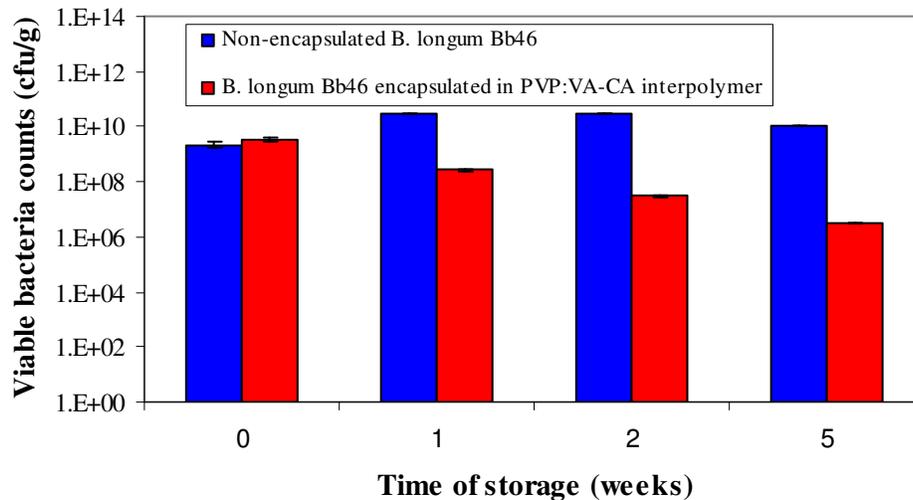


Figure 6.2: Survival of PVP:VA-VA encapsulated *B. longum* Bb-46 processed at 15 bar expansion pressure in the product chamber

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

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

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

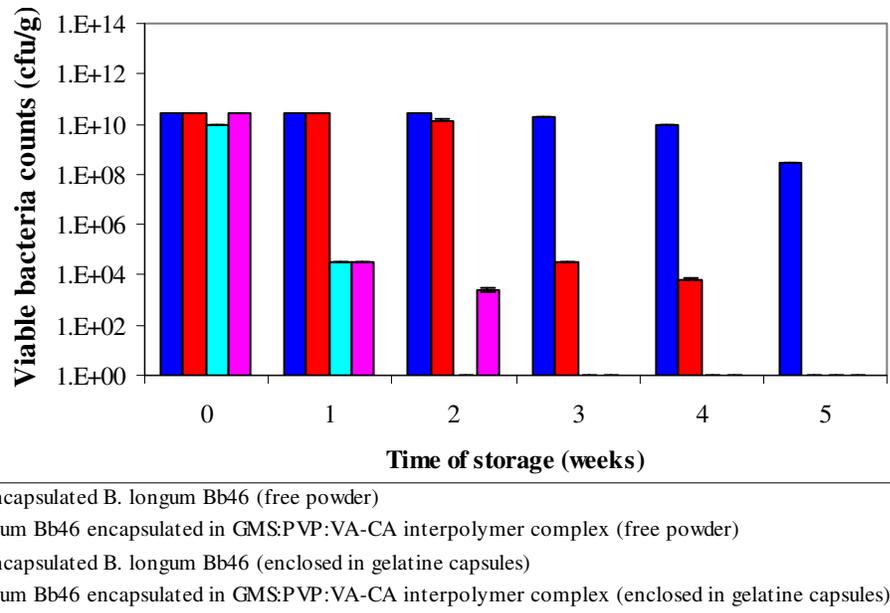


Figure 6.3: Survival of GMS:PVP:VA-CA encapsulated *B. longum* Bb-46 during storage in free powder form and when enclosed into gelatine capsules

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

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

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

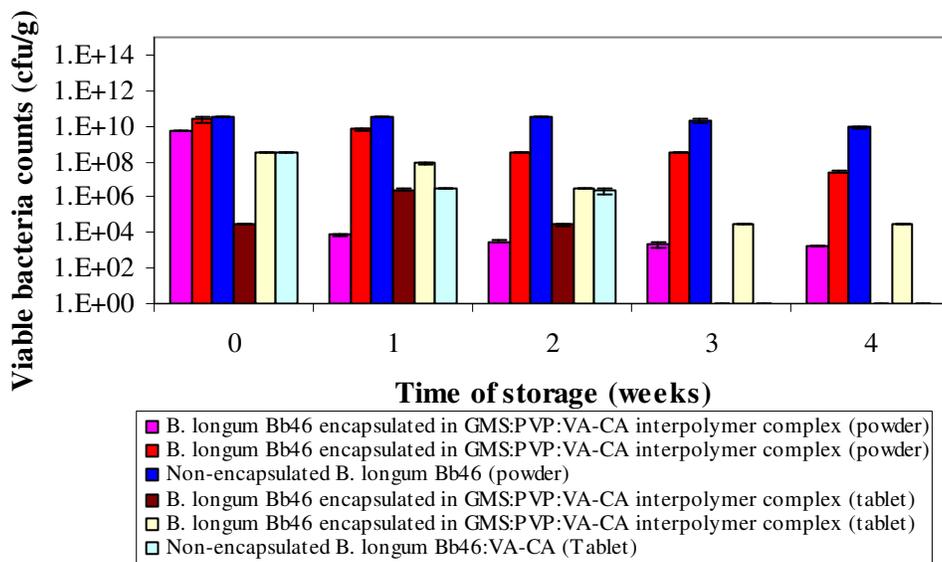


Figure 6.4 Shelf life of *B. longum* Bb-46 encapsulated in presence of GMS during storage at 30 °C in powder or tablet form

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

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

6.4.4 Effect of beeswax compared to GMS

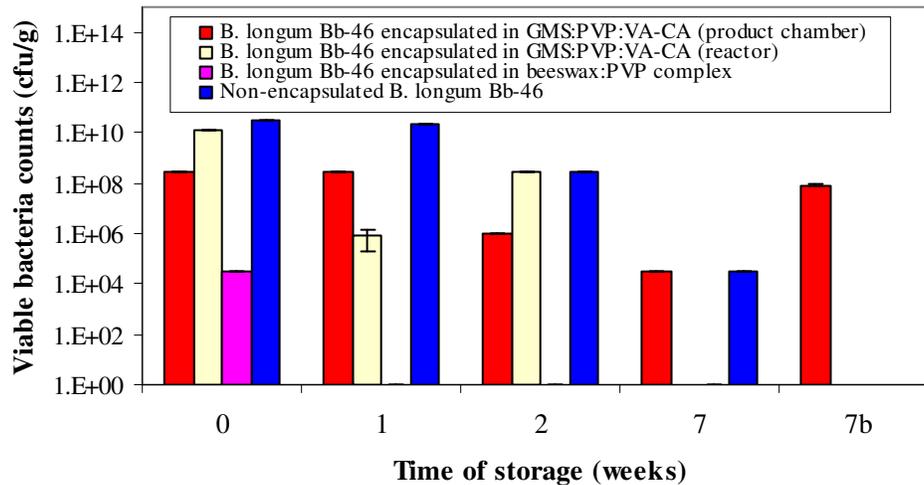


Figure 6.5: Survival of *B. longum* Bb-46 encapsulated in GMS:PVP:VA-CA and beeswax:PVP interpolymer complexes during storage at 30 °C

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

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

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

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

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

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

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

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

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

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

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

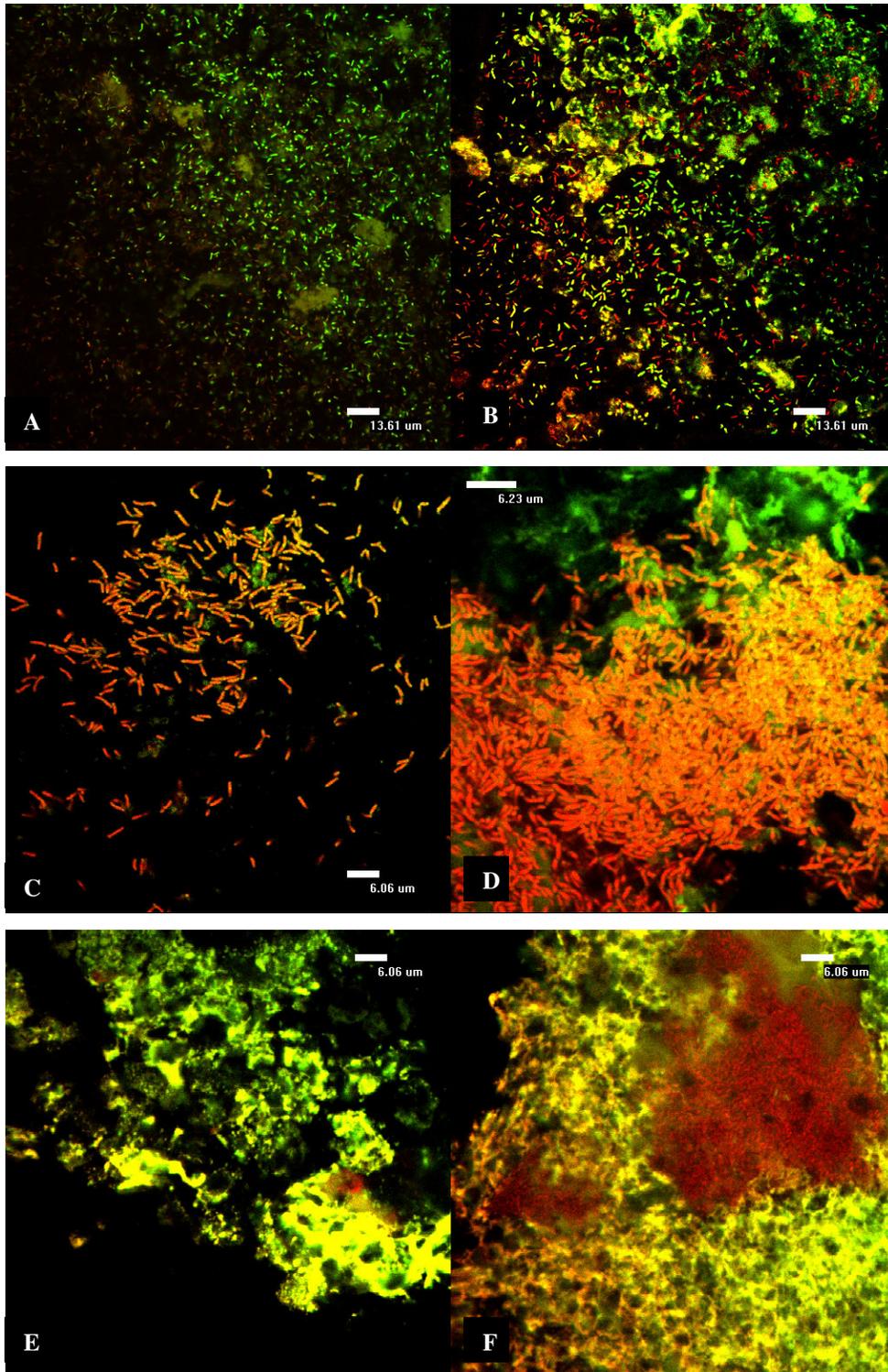


Figure. 6.6:CLSM images of Beeswax:PVP encapsulated *Bifidobacterium longum* Bb-46 stored at 30 °C for (A; B) 2 weeks, (C; D) 7 weeks and GMS:PVP:VA-CA encapsulated after storage at 30 °C for 7 weeks (E; F).

6.4.5 Water activities of the samples during storage

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

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

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

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

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

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

obtained for non-encapsulated bacteria after 10 weeks while viable counts of encapsulated bacteria were 1.65×10^6 cfu/g and 1.25×10^7 cfu/g for product chamber and reactor samples, respectively (Fig. 6.7).

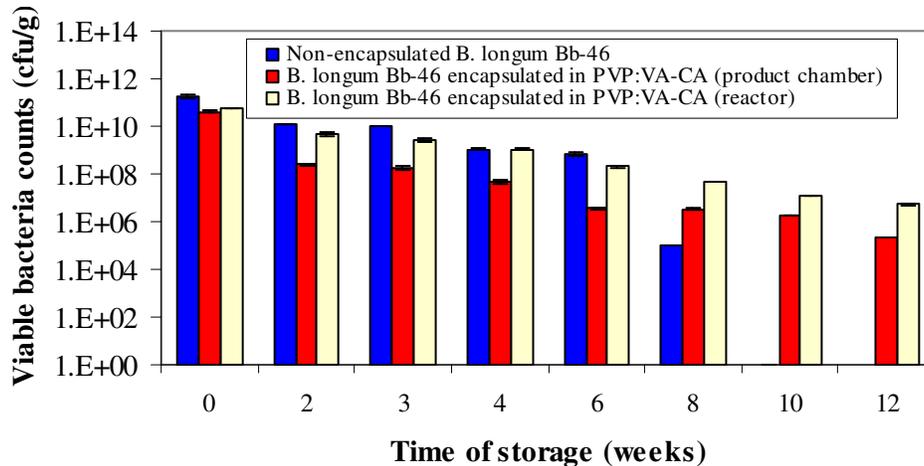


Figure 6.7: Survival of *B. longum* Bb-46 encapsulated in PVP:VA-CA interpolymers complexes after storage at 30 °C and release in SIF

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

6.5 CONCLUSIONS

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

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CHAPTER 7

GENERAL CONCLUSIONS

- South African probiotic yoghurts are not adequately labelled with regard to numbers of viable cultures in cfu/ml present in products at the end of their shelf lives, and with regards to proper identification of incorporated microorganisms up to genus and species level.
- There is generally a poor stability of probiotic bifidobacteria in products. Lactobacilli however are more stable in products and generally retain recommended levels for beneficial effects
- Scanning electron microscopy indicates that PVP and VA-CA are liquefiable in supercritical CO₂ allowing successful encapsulation of *B. lactis* Bb-12 and *B. longum* Bb-46 in the interpolymer complex
- The encapsulation process does not produce visible morphological changes to bacteria nor negatively affect viability of bacteria. High numbers of viable probiotic cultures can be encapsulated and then released from the interpolymer complex.
- The interpolymer complex has pH- responsive release properties with little to no release of encapsulated bacteria in low pH of the SGF and substantial release in high pH of the SIF. There are however batch-to-batch variations in the initial counts of encapsulated bacteria indicating different pre-exposures of the freeze-dried bacteria and with regard to release and protection of bacteria by the same interpolymer complex.
- Addition of 8 % GMS improves the protection efficiency of the basic system, PVP:VA-CA. Increasing the concentration of GMS in the interpolymer complex to as high as 60 % enhances the protective effect of the complex further.
- Alternative complexes such as PCL:VA-CA and beeswax:PVP do not protect encapsulated bacteria from the gastric acidity to the same extent as the PVP:VA-

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

- PVP:VA-CA interpolymer complex increases the shelf life of *B. lactis* Bb-12 under all storage conditions when the product is recovered from the reactor compared to when recovered from the product chamber. Encapsulation doubles the shelf life of *B. longum* Bb-46.
- Survival of bacteria is better at lower than at higher temperatures. The humidity chamber is the most unfavourable condition for storage
- The products absorb little or no moisture during storage, causing a slight change in a_w .
- Survival of bacteria is better when the product is stored in glass bottles than when stored in plastic bags. Encapsulated bacteria survive better when stored in powder form than when pressed into tablets or enclosed into gelatine capsules.
- A more representative release of bacteria from the interpolymer complex is obtained when incubation was in simulated intestinal fluid for 6 h compared to Ringer's solution.
- Encapsulation in an interpolymer complex has potential for protecting probiotic cultures from the acidity of the stomach and improving their shelf life in products.