

CHAPTER 5

Shelf life studies of *Bifidobacterium lactis*

**Bb-12 encapsulated in interpolymer
complexes in supercritical CO₂ stored
under different storage conditions**

5.1 ABSTRACT

A reduction in numbers of viable probiotic cultures in products results in lower than recommended levels for beneficial effects of these organisms in the host. Several attempts for improving survival of probiotics in products including encapsulation in different materials have been tried. However, none of the reported methods of encapsulation investigated the effect of encapsulation of probiotics in polymers in a supercritical environment. This study investigated the effect of interpolymer complex formed in scCO₂ on the shelf life of *Bifidobacterium lactis* Bb-12 under different storage conditions. The protection efficiencies of different polymer formulations were compared. These included PVP:VA-CA, PCL:VA-CA, PVP:VA-CA with GMS, addition of poly(ethylene oxide-co-propylene oxide) or PEO-PPO. Non-encapsulated and encapsulated bacteria were stored in either sterile plastic bags or glass bottles at 4 °C, 30 °C, 40 °C, 30 °C with 60 % relative humidity and room temperature. Samples were taken over time and analysed for viable bacteria. PVP:VA-CA encapsulated bacteria survived better than non-encapsulated bacteria under all storage conditions when the product was recovered from the reaction chamber. When the product was recovered from the product chamber, numbers of viable non-encapsulated bacteria were higher than the encapsulated bacteria numbers for all interpolymer complex formulations. However, it was later realized that increased release times (6 h instead of 2 h) are required to more accurately determine the total viable bacteria present in the encapsulation matrix. When these conditions were used the encapsulated probiotics always had improved shelf life compared to the non-encapsulated bacteria. The PCL:VA-CA interpolymer complex seemed weaker than the PVP:VA-CA interpolymer complex as viable counts of bacteria released from it were lower than those from the latter complex. Inclusion of PEO:PPO to both complexes had a negative effect on the protection efficiency of the matrices. Survival of bacteria was better at lower temperatures than at higher temperatures, with the humidity chamber being the most unfavourable conditions for storage.

Keywords: *Bifidobacterium lactis* Bb-12, supercritical CO₂, polycaprolactone, Glyceryl monostearate, polyethylene oxide-propylene oxide copolymer, encapsulation

5.2 INTRODUCTION

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

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

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

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

Development of a method to improve survival of probiotics in commercial products is much needed and will benefit both suppliers and consumers. This study investigated effects of encapsulation using encapsulation with an interpolymer complex in scCO₂ on survival of probiotic *B. lactis* Bb-12 under different storage conditions.

5.3 MATERIALS AND METHODS

5.3.1 Encapsulation of bacteria

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

5.3.2 Storage of samples

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

5.3.3 Enumeration of bacteria

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

release of encapsulated bacteria from the interpolymer complex matrix. Serial dilutions of the suspension were made in sterile $\frac{1}{4}$ 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).

5.4 RESULTS AND DISCUSSION

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

Since to our knowledge this work was the first to report the formation of an interpolymer complex in scCO₂ for encapsulation of probiotics, there were no similar results for comparison. However, comparison of results obtained was done with the results of studies in which probiotics were encapsulated using any other methods reported in literature. Also, most of the studies, summarized by Champagne and Gardner, 2005, investigated survival of probiotics either in milk or yoghurt (or other fermented dairy products), not when encapsulated probiotics were stored as powder as was the case in the recent study. Low levels of bifidobacteria in commercial products have been correlated with presence of starter cultures in products (Champagne and Gardner, 2005). Interference by starters has been excluded by analysing products containing only the incorporated probiotic culture.

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

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

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

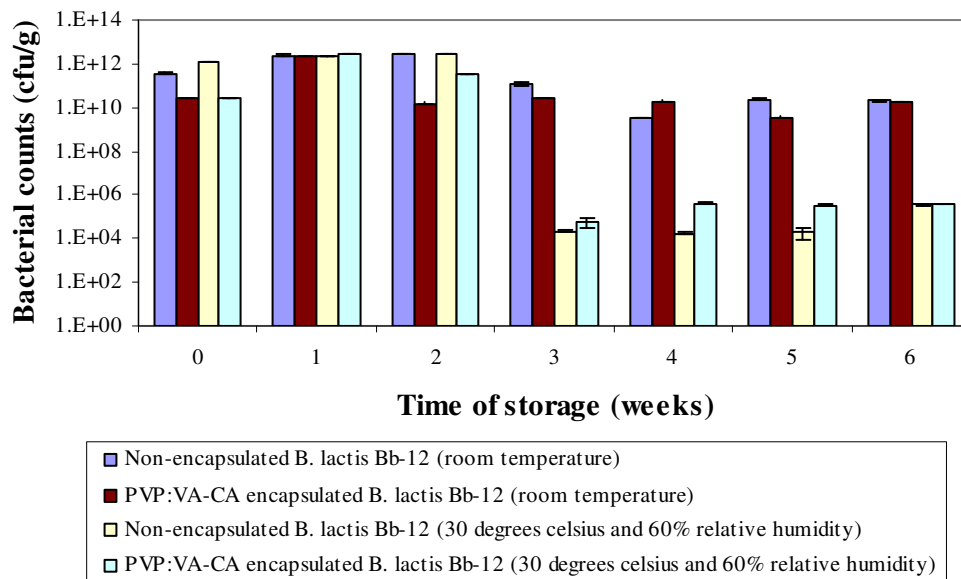


Figure 5.1: Survival of *B. lactis* Bb-12 during storage at room temperature and at 30 °C and 60 % relative humidity

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

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

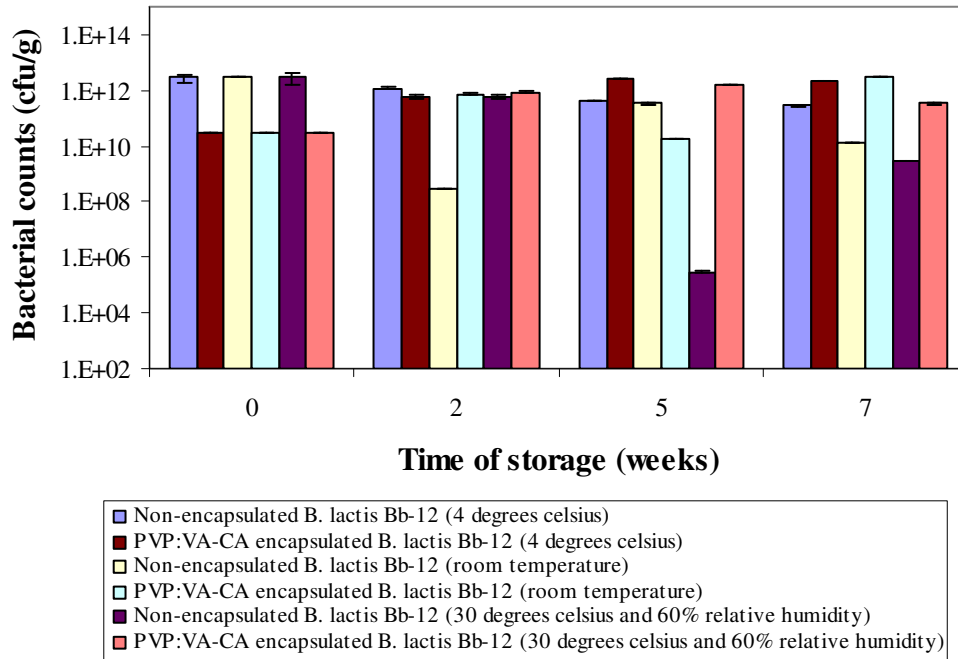


Figure 5. 2: Survival of *B. lactis* Bb-12 under different storage conditions

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

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

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

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

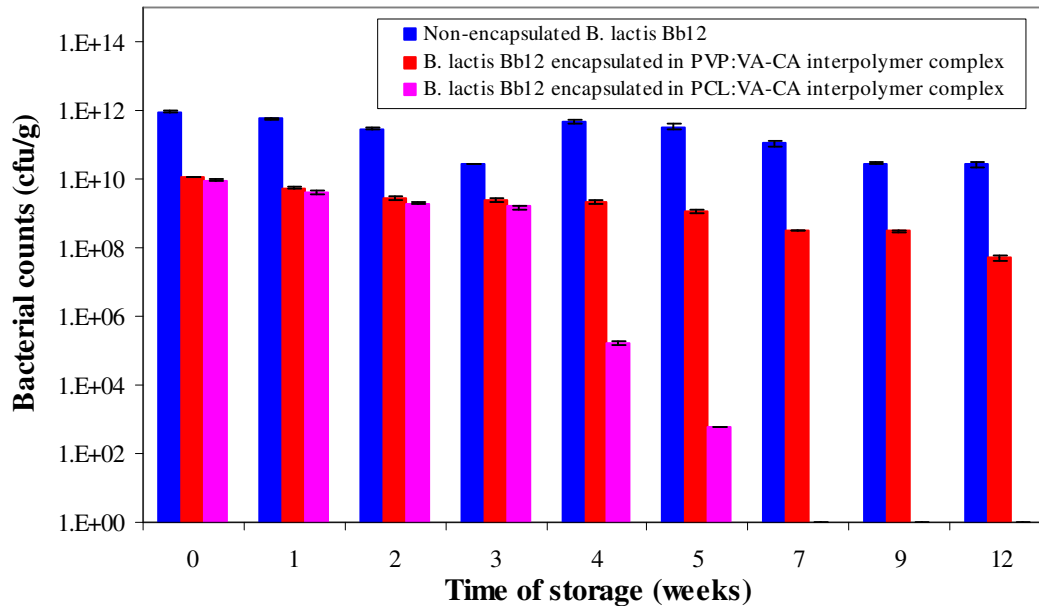


Figure 5.3: Viable counts of PVP:VA-CA and PCL:VA-CA encapsulated *B. lactis* Bb-12 over time

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

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

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

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

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

PEO-PPO was used to enhance flow in scCO₂ and aid in the formation of fine particles for encapsulated bacteria powder. When added to the PVP:VA-CA interpolymer complex, initial counts for encapsulated and non-encapsulated bacteria were the same (Fig. 5.4).

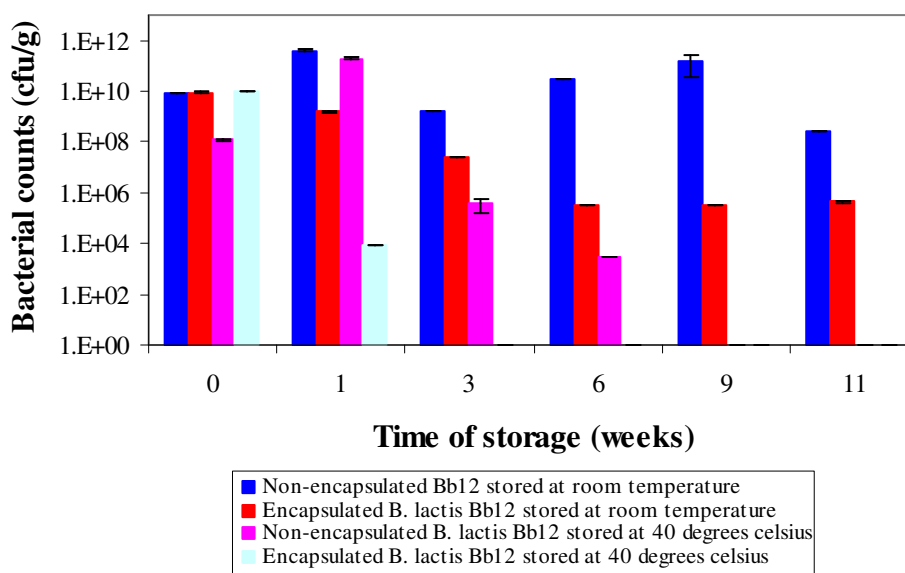


Figure 5.4: Survival of PVP:VA-CA:PEO-PPO-PEO interpolymer complex encapsulated *B. lactis* Bb-12 during storage

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

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

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

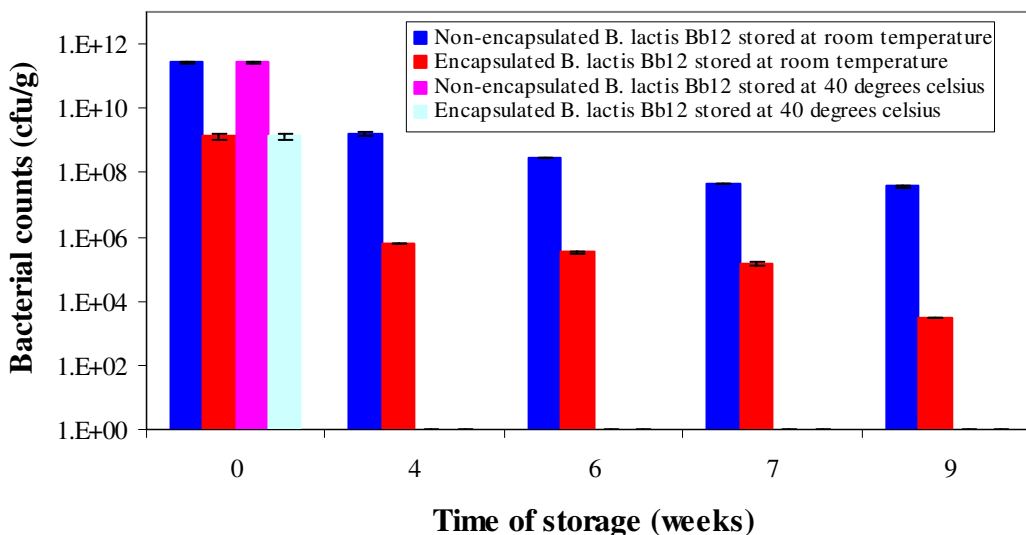


Figure 5.5. Survival of PCL:VA-CA:PEO-PPO-PEO interpolymer complex encapsulated *B. lactis* Bb-12 during storage

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

5.5 CONCLUSIONS

The PVP:VA-CA interpolymer complex formed in scCO₂ provided good protection to *B. lactis* Bb-12 when the encapsulated product was recovered from the reactor and gave negative results when the product was recovered from the product chamber after spraying through a relatively long tube with a heated nozzle at the end. The high shear process

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

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