CHAPTER 4

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

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4.1 ABSTRACT

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

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

4.2 INTRODUCTION

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

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

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

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

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

4.3 MATERIALS AND METHODS

4.3.1 Bacterial cultures

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

4.3.2 Polymer formulations

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

Table. 4.1: Polymer formulations used for bacterial encapsulation.

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

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

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

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

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

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

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

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

4.3.3 Encapsulation of bacteria

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

4.3.4 Determination of total bacteria encapsulated

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

4.3.5 Preparation of simulated gastric and intestinal fluids

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

4.3.6 Survival of bacteria in simulated gastric fluid

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

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

4.3.7 Survival of bacteria in simulated intestinal fluid

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

4.3.8 Enumeration of Bifidobacteria

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

4.4. RESULTS AND DISCUSSION

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

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

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

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

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

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

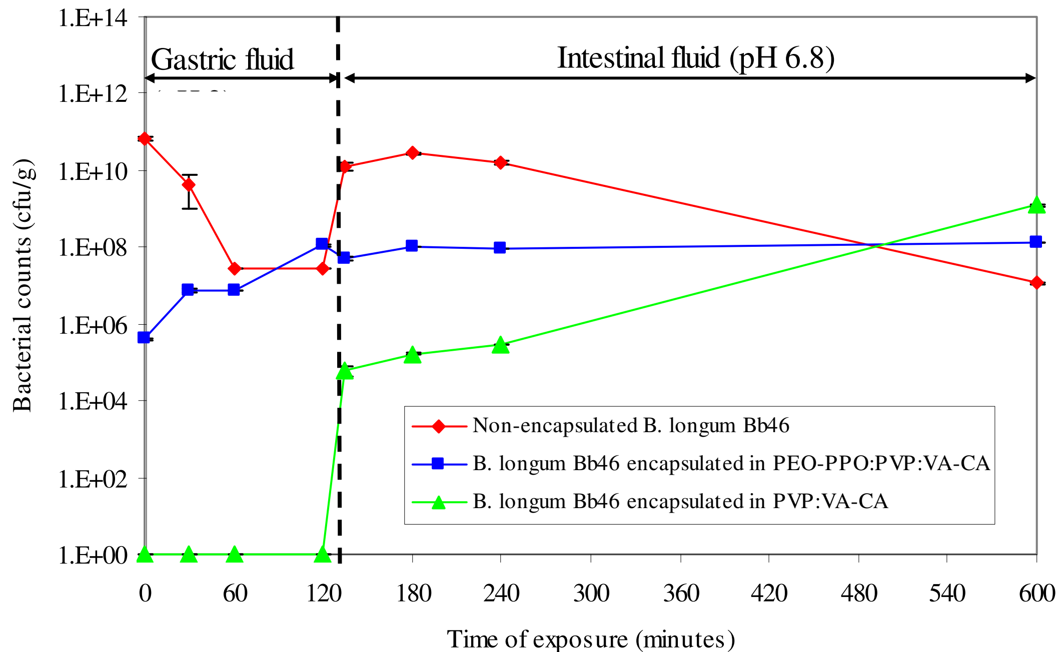


Figure 4.1: Survival of *B. longum* Bb-46 encapsulated in PVP:VA-CA and PEO-PPO-PEO:PVP:VA-CA after exposure to SGF and SIF

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

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

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

4.4.2 Survival of bacteria in polycaprolactone (PCL)

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

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

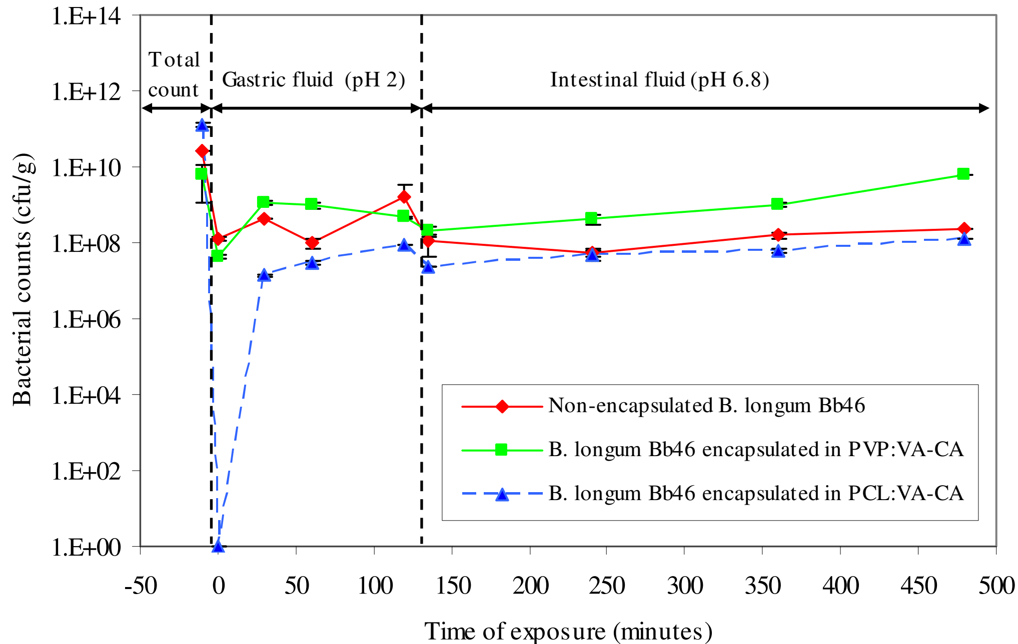


Figure 4.2: Viability of *B. longum* Bb-46 cells encapsulated in PCL:VA-CA and PVP:VA-CA after exposure to SGF and SIF

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

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

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

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

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

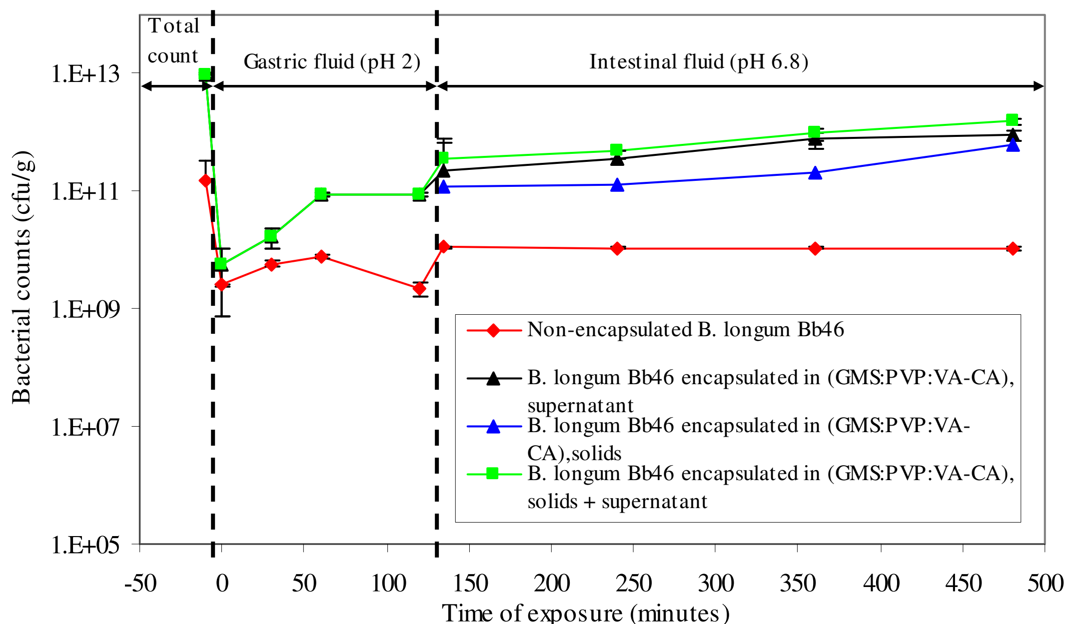


Figure 4.3: Effect of GMS incorporation into the interpolymer complex on survival of *B. longum* Bb-46 after exposure to SGF and SIF

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

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

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

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

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

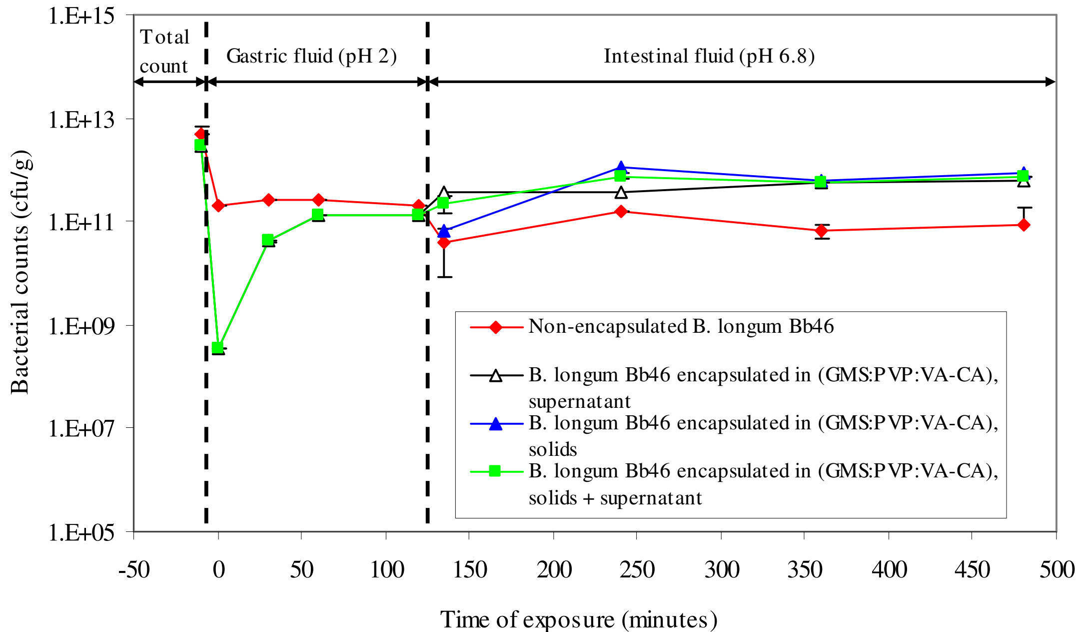


Figure 4.4: Effect of high GMS loading on survival of *B. longum* Bb-46 after exposure to SGF and SIF

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

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

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

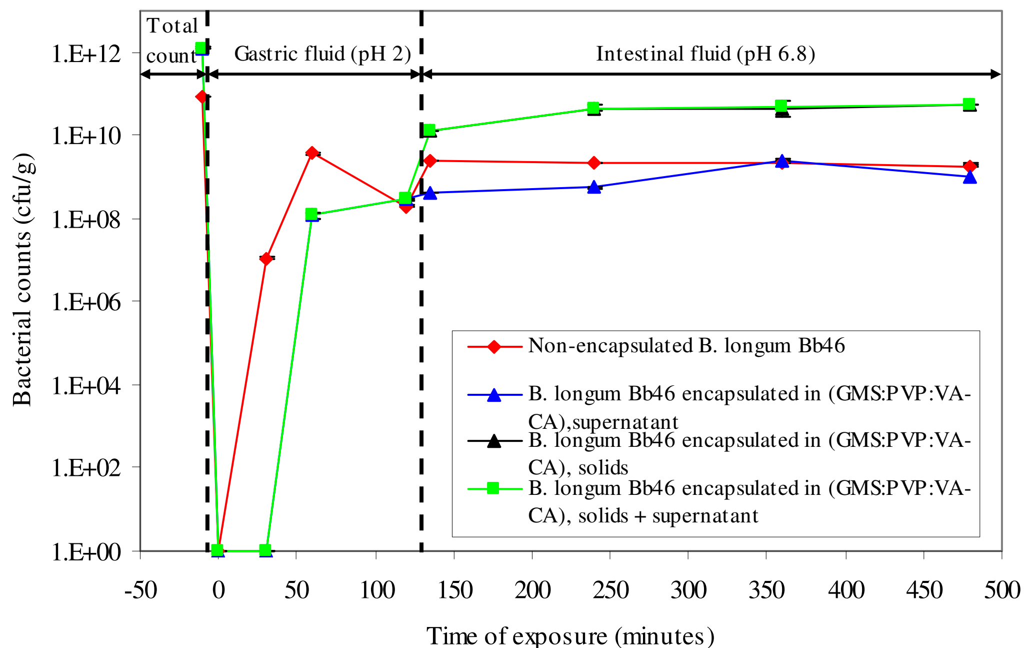


Figure 4.5: Effect of enclosure of GMS:PVP:VA-CA encapsulated *B. longum* Bb-46 into gelatine capsules on survival after exposure to SGF and SIF

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

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

4.4.6 Effect of beeswax on survival in simulated gastrointestinal fluids

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

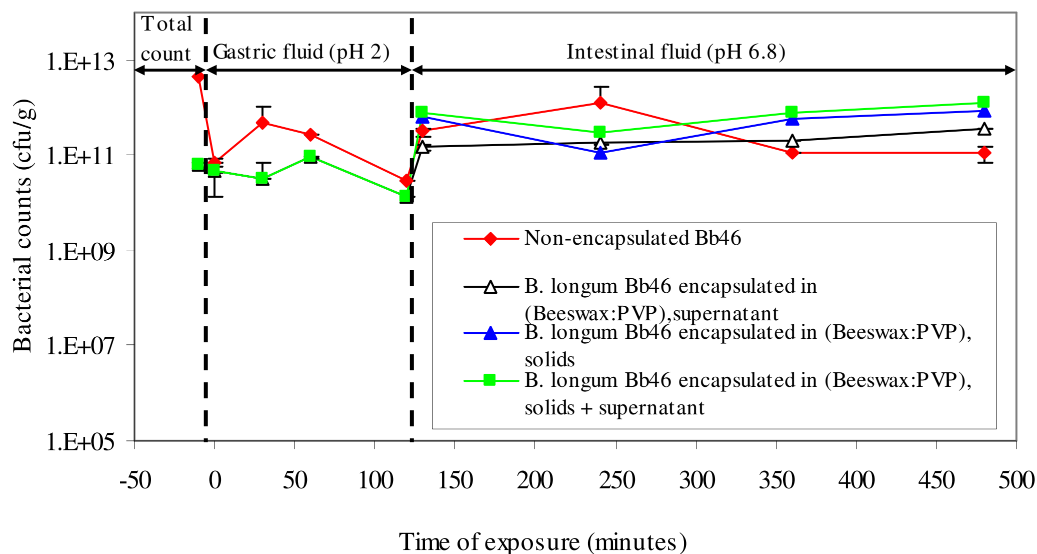


Figure 4.6: Survival of *B. longum* Bb-46 encapsulated in beeswax:PVP after exposure to SGF and SIF

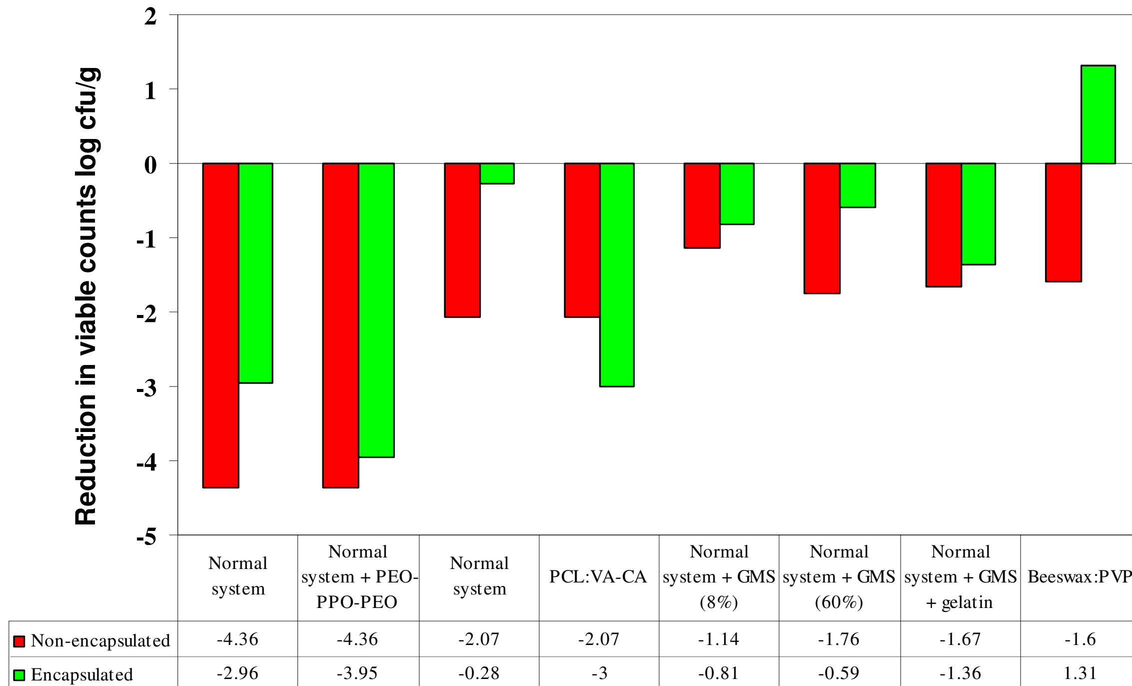
This could be due to insufficient compatibility between beeswax and PVP leading to insufficient interpolymer complex strength and/or faster release. The numbers increased over 2 h from 6.1 x 10^{10} to 1.4 x 10^{11} cfu/g (Fig. 4.6). Non-encapsulated bacteria decreased from an initial count of 1.4 x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

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

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

4.4.7 Comparing protection efficiencies of the different formulations

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



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

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

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

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

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

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

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

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

4.5 CONCLUSIONS

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

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