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Supercritical carbon dioxide interpolymer complexes improve survival of *B. longum* Bb-46 in simulated gastrointestinal fluids.

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Running title: Survival of *B. longum* Bb-46 in simulated gastrointestinal fluids

Abstract

Gastric acidity is the main factor affecting viability of probiotics in the gastrointestinal tract (GIT). This study investigated the survival in simulated gastrointestinal fluids of *Bifidobacterium longum* Bb-46 encapsulated in interpolymer complexes formed in supercritical carbon dioxide ($scCO_2$). Bacteria were exposed sequentially to simulated gastric fluid (SGF, pH 2) for 2 h and simulated intestinal fluid (SIF, pH 6.8) for 6 or 24 h. Total encapsulated bacteria were determined by suspending 1 g of product in SIF for 6 h at 37 ^{o}C prior to plating out. Plates were incubated anaerobically at 37 ^{o}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 limited reduction in viable counts at the end of exposure period due to encapsulation. Protection efficiency of the interpolymer complex was improved by addition of glyceryl monostearate (GMS). Gelatine capsules delayed release of bacteria from the interpolymer complex thus minimizing time of exposure to the detrimental conditions. Use of poly(caprolactone) (PCL), ethylene oxide-propylene oxide triblock copolymer (PEO-PPO-PEO) decreased the protection efficiency of the matrix. Interpolymer complex encapsulation showed potential for protection of probiotics and therefore for application in food and pharmaceuticals.

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Keywords: Probiotics, Interpolymer complex, *Bifidobacterium longum* Bb-46, Supercritical CO_2

1. Introduction

Several probiotic lactic acid bacteria strains are available to consumers in both traditional fermented foods and in supplement form (Kourkoutas et al., 2005). Numbers of viable organisms in products are reduced due to exposure of products to different stresses during manufacturing, storage and consumption (Doleyres and Lacroix, 2005). However, probiotic cultures must remain viable in the environment where they act, to enable them to exert beneficial effect on the consumer (Schillinger, 1999).

These organisms must therefore survive the journey through the upper GIT so that they reach the colon in large numbers to colonize the host (Kailasapathy and Rybka, 1997; Alander et al., 1999; Lian et al.,2003; Hsiao et al., 2004; Mainville et al.,2005). On arrival in the colon, the ingested probiotics compete with other bacterial species already present for nutrients and adherence sites on the intestinal epithelium (Alander et al., 1999). Viability of these cultures in the GIT is affected mainly by gastric acid present in the stomach and bile in the duodenum (Rao et al., 1989; Lo et al., 2004; Mainville et al., 2005). This sensitivity of probiotics presents a challenge for their application in different industries (Hansen et al., 2002).

Several studies have shown poor survival of many strains of bifidobacteria in acidity and bile concentration present in the human GIT. Approaches for improving survival of these bacteria include selection of acid and bile resistant strains, use of O_2 impermeable containers, two-step fermentations, stress adaptation, incorporation of micronutrients and microencapsulation (Picot and Lacroix, 2004).

Microencapsulation of bifidobacteria for improving gastrointestinal survival has been studied 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). Most results indicated improved survival. However, most of the methods present problems for large scale production though promising on a laboratory

scale (Picot et al., 2004). Also, these methods typically involve exposure of the probiotics to either water or organic solvent. This may compromise survival of encapsulated cells as they are sensitive to solvents and moisture. Thus, use of solvents should be avoided in order to improve survival. None of these previous studies reported survival of probiotics encapsulated in an interpolymer complex in supercritical CO_2 ($scCO_2$). This approach was reported for the first time by this group (Moolman et al., 2006). The aim of this study was to investigate the survival of interpolymer complex encapsulated *Bifidobacterium longum* Bb-46 in SGF and SIF, and to investigate effects of different modifications of the polymers on bacterial survival.

2. Materials and methods

2.1. Bacterial cultures

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

2.2. Polymer formulations

Different polymer formulations used for encapsulation of bacteria are summarized in Table 1.

2.3. Preparation of ingredients for encapsulation

All equipment was wiped with 70% ethanol (NCP Alcohols) using a paper towel, and allowed to dry before contact with the materials. Poly (vinylpyrrolidone) (PVP) (Kollidon 12PF, mass-average molar mass 2 000 – 3 000 g/mol, BASF) was dried for 5 h at 80 ºC and 60 mbar (absolute) in a vacuum oven (Model VO65, Vismara) and immediately placed in a dessicator to prevent moisture absorption. A sealed packet of *B. longum* Bb-46 (Chr. Hansen) was removed from storage at -12 ºC and allowed to warm to room temperature while sealed. Two grams of the bacteria was then weighed off, and ground to a powder passing through a 150 µm sieve using a coffee grinder (Model CG100, Kenwood). Vinyl acetate-crotonic acid copolymer (VA-CA) (Vinnapas C305, mass-average molar mass 45 000 g/mol, Wacker) was then weighed off and added to the bacteria, together with any additives (e.g. glyceryl monostearate – Croda Chemicals) and the dried PVP. The blend was then ground and mixed for 1 min. The amounts of the ingredients used were as in Table 1.

2.4. Encapsulation of bacteria

Encapsulation of bacteria was done as described previously (Moolman et al., 2006). Briefly, the powder blend was immediately transferred to the pre-heated 1 l reaction chamber. The chamber was then sealed, flushed and pressurized with sterile filtered CO_2 (99.995% purity, Air Products) up to a pressure of 300 bar, with the temperature controlled at 40 ºC. The material was left to equilibrate for 2 h with intermittent stirring, after which the liquefied product was sprayed through a 500 µm capillary with length 50 mm, into a 10 l expansion chamber that was pressure-controlled at 15 bar (gauge).

2.5. Preparation of simulated gastric and intestinal fluids

SGF (pH 2) was prepared according to Lian et al. (2003) while SIF (pH 6.8) was prepared according to US Pharmacopoeia (2005).

2.6. Survival of bacteria in simulated gastric fluid

One gram of either non-encapsulated bacteria or encapsulated bacteria was added to 9 ml SGF (37 ^{o}C, pH 2.0) in a test tube and vortexed for 30 s for complete dispersion. One milliliter 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. One milliliter aliquots were removed from the tubes at times 0.5, 1 and 2 h for enumeration of bifidobacteria. The test tube with encapsulated material was not vortexed during sampling so as not to interfere with release of bacteria from the interpolymer matrix. Instead, the solution was gently pipetted up and down several times before taking a sample.

2.7. Survival of bacteria in simulated intestinal fluid

One milliliter each, for non-encapsulated and encapsulated bacteria from the SGF survival test, was suspended in 9 ml of SIF (37 ^{o}C, pH 6.8) in a separate test tube and vortexed for 30 s. Excess SGF from the tube containing encapsulated material was discarded. The remaining solids were also suspended in 9 ml of SIF. One milliliter samples were taken from all the tubes immediately after suspension for enumeration of bifidobacteria. The tubes were then incubated at 37 ^{o}C in a shaker incubator, to prevent settling of released cells, at 50 rpm for 6 h. Samples were taken from the incubated tubes after 2, 4, and 6h for bifidobacteria enumeration.

2.8. Enumeration of *Bifidobacteria*

Samples were serially diluted in sterile ¼ strength Ringer's solution. Hundred microliters of appropriate dilutions were pour-plated in triplicate on MRS agar plates supplemented with 0.05% cys-HCl. The plates were incubated at 37 ^{o}C for 72 h in anaerobic jars with Anaerocult A gaspaks and Anaerocult C test strips for indication of anaerobic conditions inside the jar.

2.9. Statistical analysis

Statistical analysis was done using STATISTICA software 2008. The reported values are averages calculated from duplicate counts. Data was compared using t-test for dependent samples, samples significantly different at $p<0.05$.

3. Results and Discussion

3.1. Survival in the basic system and with added copolymer

Probiotic cultures must 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). The encapsulated probiotic bacteria were therefore exposed to SGF and SIF to investigate the potential of the encapsulation for improving survival of the bacteria under the unfavourable conditions in upper sections of the GIT. Figure 1 shows comparative counts for non-encapsulated, PEO-PPO-PEO:PVP:VA-CA encapsulated and PVP:VA-CA encapsulated bacteria during and after exposure to SGF and SIF. The non-encapsulated bacterial levels decreased during exposure to SGF. The reduction in the numbers of non-encapsulated bacteria in this study was however not as rapid as has been reported for 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. The results were however in agreement with those of the work done by Lian et al. (2003), who found that at pH 2 -3 the decrease in the number of viable bifidobacteria was not significant. No viable bacteria were released from the basic system during exposure to SGF while some release occurred from the system with added PEO-PPO-PEO. The

PVP:VA-CA matrix of the basic system did not swell or disintegrate in the acidic environment, protecting the encapsulated bifidobacteria cells from the SGF.

Release of bifidobacteria from PEO-PPO-PEO:PVP:VA-CA on the other hand indicated disintegration or swelling of the complex in the low pH environment of the SGF, to release some of the encapsulated cells. Thus, it seems that inclusion of PEO-PPO-PEO rendered the PVP:VA-CA interpolymer complex more swellable at the low pH. Hence, effectiveness of protection of bifidobacteria is reduced when PEO-PPO-PEO is used as a component in the matrix.

Numbers of viable non-encapsulated bacteria continued to decrease on subsequent exposure to SIF (Fig. 1). However, an increase in numbers of these bacteria was observed during the first 2 h exposure to SIF. A similar result was observed by Picot et al. (2004), who attributed it to temporary damage of bifidobacteria cells due to low pH stress. PVP:VA-CA interpolymer complex swelled in SIF (pH 6.8) as a result of the higher pH, releasing 9 log cfu/g bacteria after 24 h (Fig. 1). This indicated that the absence of counts from this sample in SGF was neither due to release of dead bacteria nor absence of bifidobacteria in the interpolymer matrix, but was due to the pH-dependent swellability of the matrix.

The number of viable bacteria released from the PEO-PPO-PEO:PVP:VA-CA matrix initially increased and then remained constant throughout 24 h of exposure (Fig. 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. 1). A reduction in numbers of non-encapsulated bacteria and an increase in numbers of encapsulated bacteria during the experimental period were observed with all the other formulations. PVP:VA-CA 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. PEO-PPO-PEO:PVP:VA-CA on the other hand did not protect the encapsulated bacteria from gastric acidity. Also, in the SIF an increase in the numbers of live bacteria released from this interpolymer complex was not satisfactory. Possibly, most of the bacteria from this matrix were released into and killed by the gastric fluid acidity. The normal system improved survival of bacteria ($n=18$, $p=0.047$.) more than

incorporation of PEO-PPO-PEO (n=18, p=0.170). Overall, encapsulation improved survival of bacteria ($p<0.05$).

3.2 Survival of bacteria in an alternative polymer

Polycaprolactone (PCL) is a non-hygroscopic polymer that also forms an interpolymer complex with VA-CA. It was envisaged that its non-hygroscopic nature would minimize swellability of the interpolymer complex and thus provide additional protection to the encapsulated bacteria in SGF. There was a delay in release of bacteria from the PCL:VA-CA system initially but a significant release was observed after 30 min. The delay in the release of encapsulated bacteria could be attributed to the hydrophobic nature of PCL (Pandey et al., 2005) causing slow absorption of the gastric fluid, though this desirable effect was short-lived. Also, after exposure to SIF viable counts from this system were lower than the non-encapsulated bacteria, whose viable count was lower than bacteria released from the normal system. PCL therefore seems to be a less suitable alternative than PVP even though it is non-hygroscopic (n=18, p=0.255).

3.3. Effect of GMS incorporation and gelatine capsules on survival

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. GMS (8%) was included as one of the components of the interpolymer complex. In this study both bacteria already released into SGF (supernatant) and those still retained in the interpolymer complex matrix (solid fraction) were transferred separately to SIF and analyzed. Interestingly in the SIF, there was an increase in viable counts for both the supernatant due to possible dispersion of clumps of bacteria released, and the solid fraction indicating that a significant portion of the bacteria was still retained and protected inside the interpolymer matrix. An increase in the concentration of GMS from 8 to 60% improved the protection efficiency of the GMS:PVP:VA-CA interpolymer complex. There was a significant increase in numbers of viable bacteria due to presence of GMS (n=18, p=0.045) when compared to the normal system.

Gelatine capsules are a widely accepted dosage form for delivery of probiotics via the oral route and they have been used for administration of probiotics (Saxelin et al., 1995). When bacteria were

enclosed in these capsules there was no instant release of bacteria upon exposure to SGF (pH 2) for both non-encapsulated and bacteria encapsulated in the GMS:PVP:VA-CA system. Release in SGF was delayed for 30 min and 1 h for non-encapsulated and encapsulated bacteria, respectively. The delay was longer for encapsulated bacteria due to the presence of the interpolymer complex whose swellability is restricted under low pH conditions. Gelatine capsules therefore served as an additional barrier for protection of bacteria. The increase in viability due to gelatine capsules when compared to non-encapsulated bacteria was not significant (n=18, p=0.114).

3.4. Comparing reductions in viable counts for different formulations tested over exposure period

Reduction in viable counts 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 for PCL:VA-CA and PEO-PPO-PEO systems (Fig. 2). Reduction in viable counts for the PVP:VA-CA interpolymer complex, our normal system, was not the same for 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. 2). A batch-to-batch variation in the protection and release efficiency of the same system, which still needs further attention, was thus highlighted. 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. 2). GMS alone resulted in lower reduction when compared to gelatine capsules (Fig. 2). Higher loading of GMS improved the protection efficiency further (Fig. 2). The average improvement in survival for encapsulated versus non-encapsulated *B. longum* Bb-46 was 1.61 ± 0.49 log cfu/g ($p<0.05$). There was a 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 PCL had negative effects on the properties and hence protection efficiency of the interpolymer complex.

3.5. Survival of GMS: PVP:VA-CA encapsulated bifidobacteria in SGF and SIF after storage

When the encapsulated bacteria were stored at 30 ^{o}C for seven weeks and then exposed to the SIG and SIF the results were as in Fig. 3. The results indicated that even after storage the PVP-VA-CA:GMS interpolymer matrix continued to protect the encapsulated bacteria. Viable counts obtained when

bacteria were suspended in diluent indicated higher levels of encapsulated than non-encapsulated bacteria after storage (Fig. 3). Though there were viable cells for non-encapsulated bacteria, there were no counts after exposure to SGF and SIF, indicating that the viable bacteria that were present after storage were all killed by the acidic SGF. On the contrary, for encapsulated bacteria, no cells were released during the 2 h in acidic environment of the SGF but high numbers of viable bacteria were released in SIF (Fig. 3). This is an indication that the interpolymer complex not only protected the bacteria during gastrointestinal transit, but also has the potential to improve shelf life of products containing the probiotics encapsulated in this matrix.

4. Conclusions

Encapsulation in an interpolymer complex in $scCO_2$ improved survival of *B. longum* Bb-46 through a simulated gastrointestinal environment. The encapsulation method therefore has potential for application in food and pharmaceutical industries. Future *in vitro* studies will investigate the effect of the encapsulated bacteria on the microflora of the simulator of the human intestinal microbial ecosystem (SHIME) model. The effect of encapsulation on the shelf life of probiotics will also be investigated.

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Figure Captions

Figure 1: Survival of *B*. *longum* Bb-46 encapsulated in PVP:VA-CA and PEO-PPO:PVP:VA-CA during exposure to SGF and SIF

Figure 2: Reduction in numbers of viable cells at the end of exposure period for different interpolymer complex formulations

Figure 3: Survival of *B*. *longum* Bb-46 encapsulated in GMS:PVA:VA-VA during exposure to SGF and SIF after storage

Figure 1: Thantsha et al

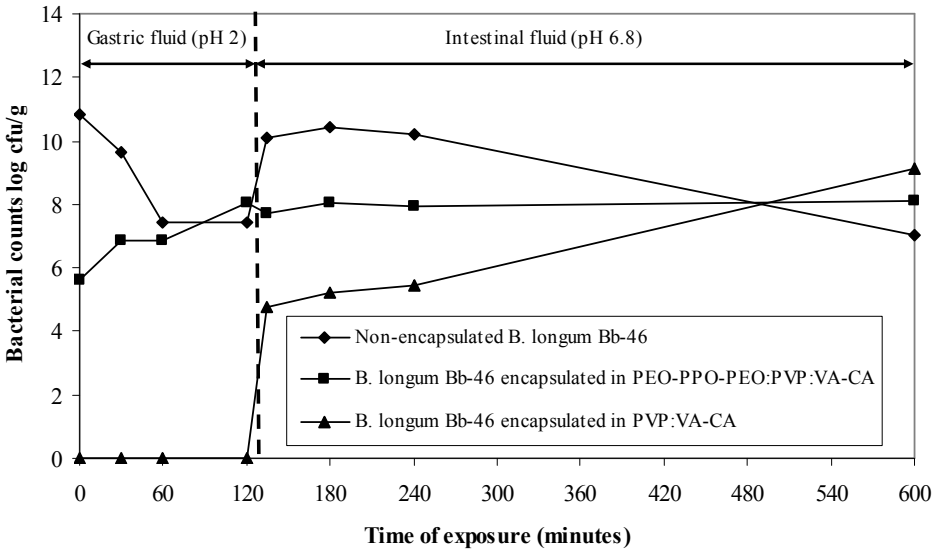
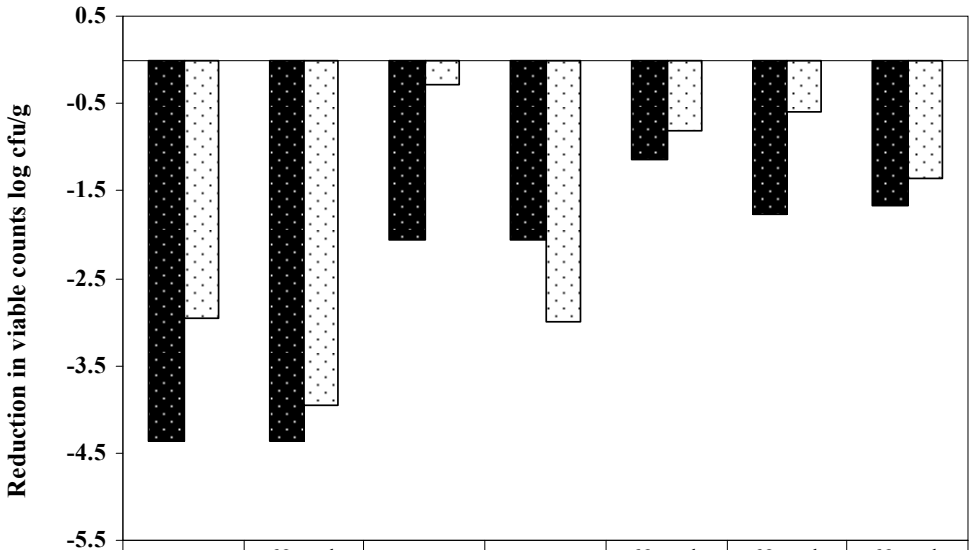


Figure 2: Thantsha et al



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

Figure 3: Thantsha et al

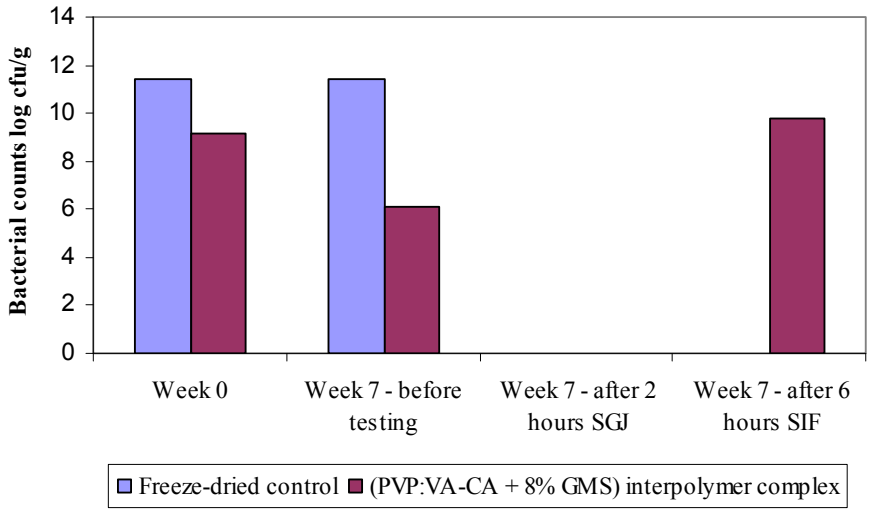


Table 1: Polymer formulations used for bacterial encapsulation.

Formulation	Different ingredients (%) w/w						Total weight (g)
	B. longum	VA-CA[a]	PVP[b]	PEO-PPO-PEO[c]	PCL[d]	GMS[e]	
1 ("basic system")	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

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

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

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

[d]PCL = Poly (caprolactone) (Tone P300- Union Carbide)

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