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Supercritical CO_2 interpolymer complex encapsulation improves heat stability of probiotic bifidobacteria

M. S. Thantsha · P. W. Labuschagne · C. I. Mamvura

Abstract The probiotic industry faces the challenge of retention of probiotic culture viability as numbers of these cells within their products inevitably decrease over time. In order to retain probiotic viability levels above the therapeutic minimum over the duration of the product's shelf life, various methods have been employed, among which encapsulation has received much interest. In line with exploitation of encapsulation for protection of probiotics against adverse conditions, we have previously encapsulated bifidobacteria in poly-(vinylpyrrolidone)-poly-(vinylacetate-co-crotonic acid) (PVP:PVAc-CA) interpolymer complex microparticles under supercritical conditions. The microparticles produced had suitable characteristics for food applications and also protected the bacteria in simulated gastrointestinal fluids. The current study reports on accelerated shelf life studies of PVP:PVAc-CA encapsulated *Bifidobacterium lactis* Bb12 and *Bifidobacterium longum* Bb46. Samples were stored as free powders in glass vials at 30 °C for 12 weeks and then analysed for viable counts and water activity levels weekly or fortnightly. Water activities of the samples were within the range of 0.25–0.43, with an average $a_w = 0.34$, throughout the storage period. PVP:PVAc-CA interpolymer complex encapsulation retained viable levels above the recommended minimum for 10 and 12 weeks, for *B. longum* Bb46 and *B. lactis* Bb12, respectively, thereby extending their shelf lives under high storage temperature by between 4 and 7 weeks. These results reveal the possibility for manufacture of encapsulated probiotic powders with increased stability at ambient temperatures. This would potentially allow the supply of a stable probiotic formulation to impoverished communities without proper storage facilities recommended for most of the currently available commercial probiotic products.

Keywords Encapsulation · Probiotics · Poly-(vinylpyrrolidone) · Bifidobacteria · Water activity

Introduction

Probiotics are live microorganisms, which when administered in adequate amounts confer a health benefit on the host (Sanders et al. 2010). Commercially, they are available in conventional fermented foods, as food supplements or food formulations and dietary supplements (Fasoli et al. 2003; Kramer et al. 2009) in capsule, liquid or powder form (Czinn and Blanchard 2009). It is generally recommended that the quantity of probiotic cultures in these products be at or above a recommended therapeutic minimum of 10^6 c.f.u./g or ml for them to elicit positive health benefits (Tamime et al. 1995; Kailasapathy and Chin 2000). Therefore, the shelf life of probiotics should be such that products with adequate live bacteria to provide health benefits are manufactured (Kourkoutas et al. 2005). A number of surveys however have indicated that cultures in products were present in levels much lower than the recommended levels at the end of shelf storage (Micanel et al. 1997; Shah 2000; Vinderola et al. 2000; Elliot and Teversham 2004; Huff 2004). Barron (1999) reported that a die-off rate of 3 log c.f.u./g occurs in probiotic products

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within 60 days of manufacture. Heat and moisture are reportedly some of the factors that cause acceleration of the die-off process (Barron 1999). Thus, the main challenge in the probiotic industry is to preserve viability of probiotics during exposure to oxygen, moisture and elevated temperatures (Jankovic et al. 2010).

Water activity (a_w) is another factor that affects shelf life of products (Fontana 2000). It is the most useful expression of water requirements for microbial growth and enzyme activity (Troller and Christian 1978). The a_w of a food describes the energy state of water in the food, and hence its potential to act as a solvent and participate in chemical or biochemical reactions. It is useful for prediction of stability and safety of food with respect to microbial growth and rates of deteriorative reactions. Water activity of different products can either increase or decrease with an increase in temperature, depending on the characteristics of the specific product (Fontana 2000). It also changes during storage with the change in the relative humidity of the environment in which the food product is stored (Adams and Moss 2008). Moisture uptake at high relative humidity, which can lead to a subsequent increase in a_w of products, continues to change as the storage time increases (Hoobin et al. 2013). It is therefore important that a_w of products during storage is monitored.

Microencapsulation has increased in popularity over the years (Stanton et al. 2005; Vidhyalakshmi et al. 2009) as a method used to create a microenvironment in which probiotics will survive during processing and storage until their targeted release (Weinbreck et al. 2010). It is important that any encapsulation method provides protection to bacteria in the products, keeping them viable throughout the storage period to ensure that consumers receive the health benefits from the ingested probiotics. We have previously reported development of a novel method for encapsulation of probiotics (Moolman et al. 2006) which produces microparticles with desirable characteristics for use in foods (Mamvura et al. 2011). We have also showed the ability of this method to protect the encapsulated probiotic bifidobacteria under simulated gastrointestinal conditions (Thantsha et al. 2009). However, the effect of this novel encapsulation method on the shelf life of encapsulated probiotics has not been reported.

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; Theunissen et al. 2005). Hence, it is worthwhile to investigate the effect of microencapsulation on different species. In this study, two commercial probiotic strains, *Bifidobacterium lactis* Bb12 with high intrinsic resistance and *Bifidobacterium longum* Bb46 with low intrinsic resistance (Booyens and Thantsha 2013), were encapsulated singly in a poly-(vinylpyrrolidone)-poly-

(vinylacetate-co-crotonic acid) (PVP:PVAc-CA) interpolymer complex using a particles from gas saturated solution (PGSS) process in which supercritical carbon dioxide is used as a solvent. The main aim was to investigate the effect of the interpolymer complex matrix on viability of encapsulated *Bifidobacterium* species during storage at 30 °C, a temperature higher than refrigerated temperature used for storage of most probiotic products.

Materials and methods

Bacterial cultures

Bifidobacterium lactis Bb12 and *B. longum* Bb46 were obtained in freeze-dried form from CHR-Hansen, Denmark. The cultures were stored at −20 °C and then used as freeze-dried powders in encapsulation experiments. Viability of the freeze-dried bacteria was determined using plate counts before encapsulation experiments.

Bacterial encapsulation

Bacterial encapsulation was carried out using the PGSS system as described previously (Moolman et al. 2006). Briefly, 2 g of PVP (Kollidon 12 PF, average molar mass 2,000–3,000 g/mol, BASF) was dry mixed with 6 g of PVAc-CA (Vinnapas C305, average molar mass 45,000 g/mol, Wacker Chemie). To this mixture 2 g of bifidobacteria was added. The dry mixed product was poured into the high pressure vessel preheated to 40 °C and then sealed. The vessel was then charged with CO_2 and pressurized to 300 bar, allowing plasticization of the PVP and PVAc-CA polymers. The system was allowed to equilibrate for 2 h and then another hour under slow stirring. After 3 h of processing, the supercritical CO_2/PVP:PVAc-CA/bacteria slurry was expanded through a 500 µM nozzle into a precipitation chamber. The rapid CO_2 expansion upon depressurization resulted in atomization of the PVP:PVAc-CA/bifidobacteria mixture combined with complexation and vitrification of the PVP:PVAc-CA polymers. In the case where the product was harvested from the reactor, after 3 h of equilibration with intermittent stirring, the reactor was depressurized and the solid polymers/bacteria mixture was removed and then ground to a fine powder using a coffee grinder. Control samples were made up of a dry mixture of freeze-dried bacteria and unprocessed polymers (i.e. polymers not processed in scCO_2). For *B. lactis* Bb12, a dry mixture of freeze-dried bacteria and polymers processed individually in the PGSS system was used as a second control, to determine whether separate processing of polymers will offer improved stability compared to unprocessed polymers.

Accelerated shelf life stability test

Encapsulated bacteria and controls were separately added into polytop glass vials and stored in an incubator maintained at 30 ± 2 °C for 12 weeks. Subsamples were taken after 7 or 14 days for water activity measurements and analysis of viability using plate counts.

Viable plate counts

Encapsulated bacteria were released from the interpolymer complex matrix using the homogenization method as described previously (Thantsha et al. 2011). A hundred microliter aliquot of the released bacteria was then serially diluted using sterile ¼ strength Ringer's solution up to 10^{-8} dilution. One gram of each control sample was suspended in ¼ strength Ringer's solution and subsequently diluted as done with released encapsulated bacteria. Then 0.1 ml of each dilution was pour plated on De Man, Rogosa and Sharpe (MRS) agar [Merck, Pty. (Ltd)], supplemented with 0.05 % cysteine hydrochloride, in triplicate. The plates were incubated at 37 °C for 72 h in anaerobic jars with Anaerocult A gas packs and Anaerotest C [Merck (Pty) Ltd] strips for maintenance and indication of anaerobic conditions, respectively. The numbers of colonies grown were counted and from these viable cells calculated and reported as colony forming units per gram (c.f.u./g).

Water activity (a_w) measurement

The water activity of all samples was measured in duplicate using the Pa$_w$Kit hand-held water activity meter according to the manufacturer's instructions. Readings were taken after 7 or 14 days over the 12 weeks of storage. The reported values are averages of two independent encapsulations.

Statistical analysis

Bacterial enumerations were done in triplicate and reported values are means of triplicate counts ± SD. Statistical analysis was conducted using student t test for independent samples using STATISTICA version 11 (StatSoft Inc, USA) and $p < 0.05$ was considered statistically significant.

Results and discussion

Viability of bifidobacteria during storage

The numbers of viable probiotic bacteria in products must be available in sufficient amounts until the products reach the end of their shelf-life. However, viable bacterial cells inevitably decrease over time, presenting a challenge in commercial probiotic production. High temperatures (>20 °C) and longer storage periods are associated with low bacterial viability (Rodrigues et al. 2011) as demonstrated by Bruno and Shah (2003), with optimal viability for long-term storage achieved at −18 °C. A temperature of 20 °C was found to result in poor viability (Bruno and Shah 2003; Meng et al. 2008). Rodrigues et al. (2011) reported better survival of *B. animalis* BB-12 encapsulated in spray dried whey protein microcapsules during storage at 5 °C than at 22 °C. Most researchers reported shelf life of probiotics stored under refrigerated conditions (Shah et al. 1995; Adhikari et al. 2000; Vinderola et al. 2000; Hansen et al. 2002). A higher temperature of 30 °C was used in this research for accelerated stability studies. Furthermore, to simulate real life storage environment, other factors that can have an influence on stability of probiotics, such as levels of oxygen and relative humidity, were not controlled. It was envisaged that should PVP:PVAc-CA interpolymer complex encapsulation retain viability of enclosed probiotics at this high temperature, then it is likely that survival at lower temperatures would also be extended. We also investigated the protection efficiency at a higher temperature with a view that, should the encapsulation method under study improve survival at this temperature, then potentially, the following additional benefits could be obtained: Firstly, improved availability of probiotics to those living in underprivileged communities without resources such as electricity. Secondly, minimized loss of probiotic viability resulting from unfavourable conditions associated with long distance transport compromising stability of probiotic cultures. Figures 1 and 2 show the changes in viability of encapsulated and unencapsulated *B. longum* Bb46 and *B. lactis* Bb12 respectively, over the 12 weeks test period.

Bifidobacterium longum Bb46

Samples from both the reactor and product chamber were analysed in the shelf life studies of *B. longum* Bb46 to determine the effect of the atomization process on the stability of the bacteria. The CO_2/polymer/probiotic slurry has a relatively high viscosity and during the atomization process from the reactor to the product chamber, it is exposed to high shear forces and heating to counter the cooling effect of expanding CO_2. These conditions could be detrimental to probiotics, especially the more sensitive strains, and it is thus important to determine if they cause a difference in stability of final products obtained from the two chambers.

Viable counts of encapsulated *B. longum* Bb46 were higher for the encapsulated product harvested from the reactor than that from the product chamber. Unencapsulated bacteria decreased by 6 log c.f.u./g after 8 weeks of
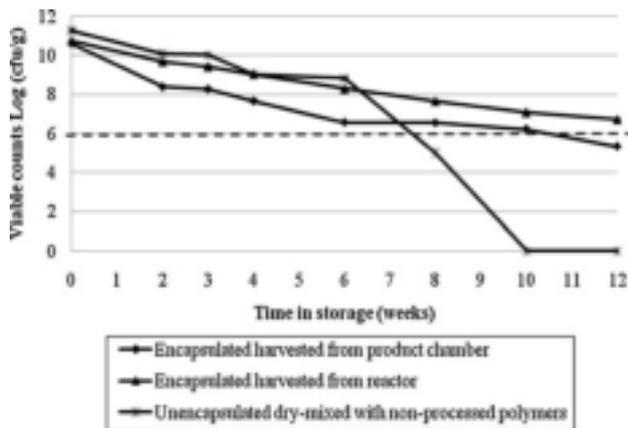
Fig. 1 Survival of unencapsulated and PVP:PVAc-CA interpolymer complex encapsulated *B. longum* Bb46 during 12 weeks of storage at 30 °C. *Each point* represents the average of triplicate counts from three independent experiments and *error bars* (less than 0.1, hence invisible on *graph*) represent the SD of 3 replicates. The *dotted line* represents the minimum recommended level for probiotic viability
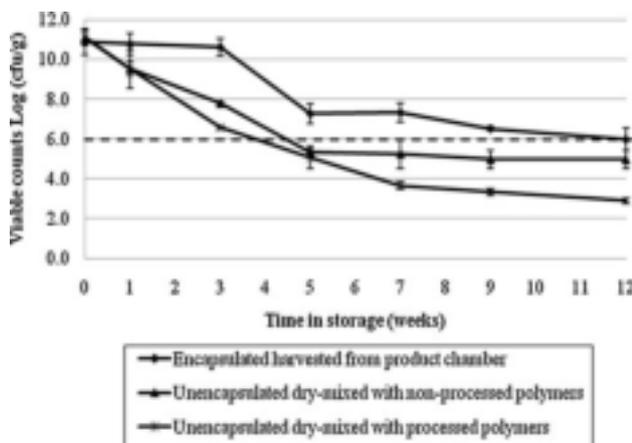

Fig. 2 Survival of unencapsulated and PVP:PVAc-CA interpolymer complex encapsulated *B. lactis* Bb12 during 12 weeks of storage at 30 °C. *Each point* represents the average of triplicate counts from three independent experiments and *error bars* represent the SD of 3 replicates. The *dotted line* represents the minimum recommended level for probiotic viability

storage at 30 °C while the encapsulated bacteria decreased by 4 and 3 log c.f.u./g for product harvested from the product chamber and reactor, respectively. No viable counts were obtained for unencapsulated bacteria after 10 weeks while viable counts of encapsulated bacteria were 6 and 7 log c.f.u./g for product chamber and reactor samples, respectively, after the same period (Fig. 1). This indicated that all the bacteria that were present in the unencapsulated sample lost viability between weeks 8 and 10. It is worth noting that even though harvesting of the encapsulated bacteria from the product chamber resulted in more reduction of viable counts than the reactor-harvested product, viability levels of bacteria in this product

remained higher than that of unencapsulated bacteria from 8 weeks until the end of the storage period. Unencapsulated bacteria had acceptable levels of viable bacteria (level above the recommended minimum of 6 log c.f.u./g) up to 6 weeks of storage while the encapsulated bacteria maintained this level for up to 10 and 12 weeks for product chamber and reactor samples, respectively (Fig. 1). Thus, encapsulation increased shelf life of *B. longum* Bb46 by 4 weeks when the encapsulated bacteria were harvested from the product chamber and by 6 weeks when recovered from the reactor. The viable counts for the product harvested from the product chamber were somewhat higher ($p = 0.05$) than the unencapsulated cells while the counts for product harvested from the reactor were significantly higher ($p < 0.05$) than the unencapsulated bacteria. These results indicated that exposure of bacteria to high shear during spraying into the product chamber seems to have a negative effect on bacterial viability, highlighting that this loss in viability should be compensated for during formulation. However, regardless of the observed higher viability obtained with the reactor-harvested bacteria, the encapsulated bacteria used in further trials were recovered from the product chamber. Harvesting of the encapsulated bacteria from the reactor will not only be impractical for large scale production, but would also increase the chances of product contamination.

Bifidobacterium lactis Bb12

Encapsulated bacteria showed a 5 log c.f.u./g reduction in viability over the test period, whereas unencapsulated bacteria dry-mixed with unprocessed polymers and those dry-mixed with PGSS processed polymers decreased by 6 and 8 log c.f.u./g, respectively (Fig. 2). The highest reduction in viable counts for all the samples occurred between weeks 3 and 5, when there was a 3.3 log c.f.u./g decrease in viability of encapsulated bacteria, and about 2.5 and 1.5 log c.f.u./g decrease for unencapsulated bacteria dry-mixed with unprocessed polymers and those dry-mixed with processed polymers, respectively (Fig. 2). It was noted that it was also during this period that the highest water activities were recorded (Table 1). Elevated water activities negatively affect viability of bacteria and therefore, this increase in water activity could have also contributed to viability losses. By week 5 viability levels of both samples of unencapsulated bacteria had dropped below the recommended minimum while viable numbers for encapsulated bacteria remained above this level. Contrary to what was observed for unencapsulated *B. longum* Bb46, there were viable cells for unencapsulated *B. lactis* Bb12 until 12 weeks of storage, though the levels were below the recommended minimum from week 5 onwards (Fig. 2). This suggests that *B. lactis* Bb12 is intrinsically more stable under unfavourable

Table 1 Water activities of samples stored at 30 °C for 12 weeks

Sample descriptions	Number of weeks in storage					
	0	1	3	5	7	12
B. lactis Bb12	Water activity measurement					
Unencapsulated (with unprocessed polymers)	0.27 ± 0.06	0.32 ± 0.01	0.37 ± 0.02	0.31 ± 0.03	0.25 ± 0.01	0.21 ± 0.02
Unencapsulated (with processed polymers)	0.28 ± 0.05	0.37 ± 0.02	0.39 ± 0.02	0.31 ± 0.04	0.24 ± 0.02	0.21 ± 0.01
Encapsulated (product chamber)	0.34 ± 0.01	0.37 ± 0.03	0.41 ± 0.01	0.36 ± 0.02	0.30 ± 0.01	0.30 ± 0.01
B. longum Bb46						
Unencapsulated (with unprocessed polymers)	0.25 ± 0.02	0.28 ± 0.1	0.41 ± 0.01	0.39 ± 0.03	0.40 ± 0.02	0.40 ± 0.02
Encapsulated (product chamber)	0.35 ± 0.05	0.40 ± 0.03	0.43 ± 0.03	0.43 ± 0.02	0.39 ± 0.02	0.41 ± 0.01
Encapsulated (reactor)	0.22 ± 0.1	0.29 ± 0.01	0.41 ± 0.04	0.39 ± 0.02	0.39 ± 0.02	0.40 ± 0.02

Data are expressed as averages ± SD of duplicates from two independent experiments

conditions than *B. longum* Bb46. However, the counts for encapsulated bacteria were significantly higher ($p < 0.05$) than those for both unencapsulated samples at the end of storage. Encapsulation therefore increased survival of *B. lactis* Bb12 probiotic from 5 to 12 weeks.

Interestingly, lower viability levels were obtained throughout the storage period for both unencapsulated *B. lactis* samples when compared to the encapsulated sample. This observation further highlights that protection is only offered when bacteria are entrapped within the interpolymer complex matrix. Thus, the interpolymer complex formed through interaction of polymers when both are present under supercritical conditions, via hydrogen bonding between the carbonyl group of PVP and the carboxyl group of PVAc-CA (Moolman et al. 2006), is critical for protection of bacteria.

Comparison of total reductions in viability of bacteria during storage

Figure 3 shows the total reduction in viability of the *Bifidobacterium* cells in encapsulated and unencapsulated samples at the end of storage at 30 °C for 12 weeks. Calculating the differences between the initial viable counts and the count obtained at the end of storage, it was observed that encapsulation improved viability of both *Bifidobacterium* species tested. However, the benefit of encapsulation was more for *B. longum* Bb46 than for *B. lactis* Bb12. Looking at *B. longum* Bb46, the levels of unencapsulated bacteria decreased by 11 log c.f.u./g while those of encapsulated bacteria decreased by 5 log c.f.u./g (Fig. 3). Thus, encapsulation increased survival of *B. longum* Bb46 by 6 log c.f.u./g under the test conditions, i.e. elevated temperature, to which the bacteria were exposed during storage. Similarly, unencapsulated *B. lactis* Bb12 cells decreased by 8.2 log c.f.u./g while encapsulated cells decreased by 4.9 log c.f.u./g (Fig. 3), which indicated improved survival of 3.3 log c.f.u./g over a 12 week
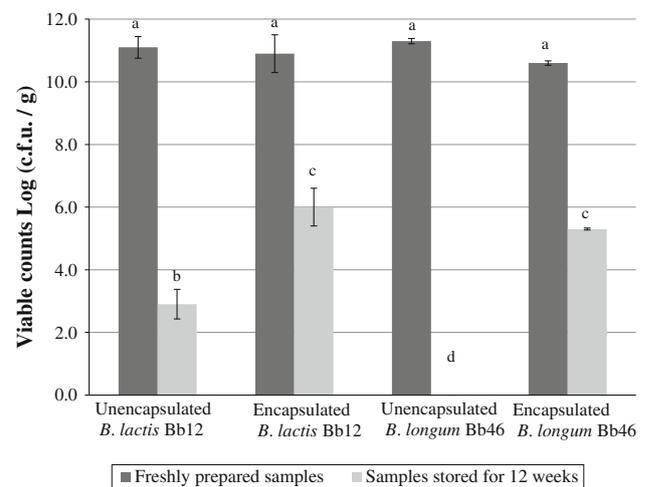

Fig. 3 Comparison of viable counts for freshly prepared unencapsulated (bacteria mixed with unprocessed polymers) and their encapsulated counterparts (harvested from product chamber) stored for 12 weeks at 30 °C. *Each bar* represents the average of triplicates from three independent experiments and the *error bars* are SD. *Different letters* denotes significant differences in total reduction in viable counts at the end of storage

period. The results obtained further indicated that different *Bifidobacterium* species will be affected differently by the encapsulation process. For both *Bifidobacterium* species tested, the total reduction in viable counts for encapsulated samples at the end of storage period was significantly lower ($p < 0.05$) than that for unencapsulated samples.

Researchers elsewhere reported on encapsulation of bifidobacteria mostly in yoghurt and in other products at lower storage temperatures. Increase in survival of encapsulated bifidobacteria added to yoghurt was reported to range between 0.5 and 1 log cycle for storage period of between 1 and 7 weeks for different *Bifidobacterium* species (Heidebach et al. 2012). Boylston et al. (2004) reported loss of 3 log c.f.u./g for *B. infantis* and *B. breve* within 14 days of refrigerated storage.

Increased survival of different *Bifidobacterium* species encapsulated using a variety of coating materials and encapsulation techniques ranged between 0.5 and 2 log - cycles with storage periods between 2 and 12 weeks (Heidebach et al. 2012). The highest improvement was survival of 5 log cycles which was reported for *B. bifidum* and *B. infantis* encapsulated separately in alginate and stored in mayonnaise for 8 weeks (Khalil and Mansour 1998). It should also be noted that some authors showed no improvement of viability after encapsulation (Khalil and Mansour 1998; Heidebach et al. 2012). Although survival of probiotics in food products is important, maintaining their viability in dry powders during storage is considered an important determinant of the product's commercial success (Simpson et al. 2005). It is for this reason that, in this study, storage stability of encapsulated probiotics in dry powder form was measured. Lee et al. (2004) reported increased survival of 2 log cycles for *Lactobacillus bulgaricus* KFRI 673 encapsulated in chitosan coated calcium alginate microparticles stored at 22 °C for 4 weeks. On the other hand, Simpson et al. (2005) spray dried *Bifidobacterium* species in reconstituted skimmed milk containing gum acacia, and reported viability losses of 4 and >8 log c.f.u./g after storage of powders for 90 days at 25 °C for *B. animalis* ssp. *lactis* BB12 and *B. longum* biotype *longum* NCIMB 8809, respectively. It is worth noting that in our study, storage stability tests of encapsulated bifidobacteria microparticles were conducted at even higher temperature, and most importantly, increased survival due to PVP:PVAc-CA encapsulation was obtained for both *Bifidobacterium* species tested.

Water activity values of samples during storage

Water activity is a crucial factor to consider in maintaining probiotic viability over the expected shelf-life for therapeutic benefits to be realized (Weinbreck et al. 2010). It is also better to show the relationship between bacterial survival and changes in the mobility of water in their microenvironment, than a correlation to a_w of the environment (Hoobin et al. 2013). It has been reported that elevated water activity levels above 0.25 affect viability of microorganisms negatively (Teixeira et al. 1995; Weinbreck et al. 2010). Abe et al. (2009) found the effect of a_w to be the strongest factor in decreasing probiotic viability. The best viability seems to be maintained by powders with a water activity of 0.2, which is equivalent to 4 % moisture content (Simpson et al. 2005).

In the current study, low water activities of between 0.25 and 0.43, with an average of 0.34, were obtained for all the test samples for most of the duration of their storage at 30 °C (Table 1). These levels were similar to those recorded previously for microcapsules encapsulating *B.*

infantis (Crittenden et al. 2006). These researchers however only reported initial a_w values of their microcapsules. It is therefore unknown whether the reported values increased, decreased or remained the same during the storage period. In the current study, a_w values of the samples increased at the beginning of storage but then decreased again towards the end. The highest water activities obtained for encapsulated bacteria were 0.41 and 0.43 for *B. lactis* Bb12 and *B. longum* Bb46, respectively. These levels were obtained after storage of samples for 3 weeks, and were accompanied by a high reduction in bacterial viability as mentioned earlier. However, though cell death was high during this period, the levels of viable cells remained above the recommended minimum for beneficial effects.

It was interesting to observe that although unencapsulated bacteria had lower a_w than encapsulated samples, they displayed higher loss in viability compared to their encapsulated counterparts. This suggests that a_w was not the only factor causing a reduction in cell viability, but that other factors also contributed to bacterial death. It also suggests that the benefits provided by the interpolymer complex overrides the detrimental effect of a higher a_w. All samples were stored in glass vials, which could also have aided in shielding them from the negative effects of oxygen toxicity. Better survival of bifidobacteria stored in glass than polyester bottles was reported previously (Dave and Shah 1997; Hsiao et al. 2004). Glass has also been shown to improve survival of bacteria when compared to poly (ethylene terepthalate) (PET) bottles (O'Riordan et al. 2001). PET has higher oxygen permeability than glass (Ishibashi and Shimamura 1993; Hsiao et al. 2004). However, since all samples in this study were stored in glass, improved probiotic viability can be attributed mainly to the protective effect of the interpolymer complex.

Water activity values for all the samples were much lower than 0.86 and 0.9 which are the lowest a_w values at which most spoilage bacteria grow under aerobic and anaerobic conditions, respectively (Fontana 2000). With the exception of *Staphylococcus aureus* which can grow at a_w levels as low as 0.86, most pathogens and other bacteria require a_w above 0.94 (Cloete and Atlas 2006). Therefore, growth of the majority of spoilage microorganisms in the encapsulated bacteria powder will be very minimal or absent. This will help with regard to safety of the probiotic product.

Conclusions

PVP:PVAc-CA interpolymer complex encapsulation appreciably improved viability of bifidobacteria at 30 °C. The benefit of encapsulation was most pronounced for *B. longum* Bb46, with lower inherent stability, than for *B.*

lactis Bb12, the intrinsically more stable species. This method of encapsulation reveals the possibility for manufacture of encapsulated probiotic powders with increased stability at ambient temperatures. This would potentially allow the supply of a stable probiotic formulation to impoverished communities without proper storage facilities recommended for most of the currently available commercial probiotic products. Since viability was done on microparticles stored in powder form, there is a need for stability of the product to be further tested when incorporated into foods to establish whether similar protection will be obtained.

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