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RESEARCH ARTICLE

Improved viability of *Lactobacillus acidophilus* NRRL-B 4495 during freeze-drying in whey protein-pullulan microcapsules

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Abstract

In this research, pullulan was incorporated in protein-based encapsulation matrix in order to assess its cryoprotective effect on the viability of freeze-dried (FD) probiotic *Lactobacillus acidophilus* NRRL-B 4495. This study demonstrated that pullulan in encapsulation matrix resulted in a 90.4% survival rate as compared to 88.1% for whey protein (WPI) encapsulated cells. The protective effects of pullulan on the survival of FD-encapsulated cells in gastrointestinal conditions were compared. FD WPI-pullulan capsules retained higher survived cell numbers (7.10 log CFU/g) than those of FD WPI capsules (6.03 log CFU/g) after simulated gastric juice exposure. Additionally, use of pullulan resulted in an increased viability after bile exposure. FD-free bacteria exhibited 2.18 log CFU/g reduction, while FD WPI and FD WPI-pullulan encapsulated bacteria showed 0.95 and 0.49 log CFU/g reduction after 24 h exposure to bile solution, respectively. Morphology of the FD microcapsules was visualized by scanning electron microscopy.

Keywords

Pullulan, whey protein, encapsulation, probiotic

History

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Introduction

Due to long storage periods or difficulties in handling large volumes, bioactive compounds including probiotics are generally preferred to be used in dehydrated forms (Carvalho et al., 2004; De Giulio et al., 2005; Su et al., 2007). Freeze drying also known as lyophilization is the most commonly used drying method for preserving sensitive bioactive compounds during the preparation of solid particles. This technique is based on removing the moisture from the medium by sublimation at ambient temperature and thereby exerting a very minimum heat stress to the living cells. However, in the literature, there are few studies conducted on viability loss of probiotic bacteria after freeze drying. These studies indicated that additional action had to be taken to overcome the negative sides of freeze drying. Further studies focused on the use of sugars as cryopreservatives and micro-encapsulation methods to avoid these effects (Kumar and Mishra, 2004; Peighambardoust et al., 2011).

Capela et al. (2006) studied the effect of microencapsulation and cryoprotectants on the survival of four probiotic species in freeze-dried (FD) yoghurt and obtained an improved viability when microencapsulated in alginate beads. In another study, casein-based microcapsules were reported to improve the viability of *Lactobacillus paracasei* ssp. *paracasei* F19 cells significantly (lost about 10% in survival rate), whereas free cells lost the viability about 30% after freeze drying (Heidebach et al., 2010). These studies also showed that the survival rates of probiotic bacteria changes depending on the added cryoprotectants and microencapsulation techniques/materials.

Whey protein is one of the most widely used polymer for microencapsulation processes. It is proved to be an effective encapsulating agent in microencapsulation of bioactive ingredients because of their ability to form microcapsules easily under mild conditions using different techniques. Combination of whey protein with different sorts of polysaccharides has been studied for microencapsulation of bioactive substances. Among these polysaccharides, pullulan is not extensively studied for the microencapsulation of probiotics. It has been most widely searched to form films with proteins including whey protein and soy protein due to its non-toxic, water-soluble, colourless, tasteless, odourless and heat stable characteristics.

The aim of this article was to study the cryoprotective effect of pullulan in protein microencapsulation matrix on the survival of probiotic bacteria during freeze drying. Also, survival of free and microencapsulated FD bacteria during storage and in *in-vitro* gastrointestinal conditions was observed.

Materials and methods

Materials

Commercial strain of *L. acidophilus* NRRL-B 4495 was obtained from the ARS Culture Collection (NRRL, Peoria, IL). Ox-bile was obtained from Fluka Biochemica (Switzerland). Trypsin (from bovine) was purchased from Merck (Darmstadt, Germany) and pepsin (from porcine stomach mucosa) from Sigma (St. Louis, MO). Sunflower oil was obtained from a local store. Pullulan was a gift from Hyarashiba Co. (Japan). Whey protein isolate (WPI) was obtained from BiPro, Danisco, Denmark. All the other chemicals were obtained from Sigma (St. Louis, MO).

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Methods

Bacterial strain and culture preparations

Lactobacillus acidophilus NRRL-B 4495 cells were inoculated into 5 ml of MRS broth and incubated at 37 °C for 24 h under anaerobic conditions. The cultures were then subcultured into 20 ml of MRS broth and incubated under same conditions for 12 h. The cells were harvested by centrifugation at 15,000×*g* for 10 min at 4 °C from 20 ml of a 12-h culture in the late log phase. The supernatant was decanted, and the cells were re-suspended in 100 ml of pullulan-WPI solution obtaining a cell load of about 8.5 log CFU ml^{-1}.

Formation of WPI-pullulan wall matrix

WPI-pullulan microcapsules were prepared according to the method of Wood (2010) with some modifications. Briefly, WPI (9% w/v) was dispersed by mixing the protein powder in sterile distilled water at an ambient temperature. Protein solution was stirred for approximately 3 h using a magnetic stirrer to ensure proper dissolution under 4 °C, and after hydration protein solution was denatured at 80 °C for 30 min. Denatured protein solution was cooled to room temperature in an ice bath. Pullulan in varying concentrations were dissolved in distilled water at an ambient temperature, and pullulan solution was stirred for approximately 3 h using a magnetic stirrer to ensure proper dissolution. Pullulan solution was then sterilized using 0.45 μm filter and mixed with the denatured WPI solution at a concentration of 9% (w/v).

Preparation of microcapsules

In the first step, primary water-in-oil emulsions (W_1/O) were formed by emulsifying an inner aqueous phase (W_1) made up by WPI-pullulan containing bacteria into an oil phase (O). W_1/O emulsion was prepared by an Ultra Turrax homogenizer (3000 rpm for 5 min, Ultra Turrax, model T25, Janke & Kunkel, IKA Labortechnik, Staufen, Germany). This emulsion was homogenized in $CaCl_2$ solution (100 mM) for 2 min at 3400 rpm. After formation of the microcapsules, this slurry was orbitally shaked at 160 rpm for 30 min to harden the microcapsules. The hardened microcapsules were separated from the solution and oil phase by two sets of homogenization at 1000 rpm for 1 h. It is important to state that in order to avoid the destructive heat generation possibly formed during homogenization, microencapsulation process was conducted inside the ice bath.

Freeze drying and bacterial enumeration

Microcapsules or free bacterial solution were frozen at −20 °C. Thereafter, the beads were freeze-dried in a Lablanco freeze-dryer (Freezone 18, Kansas, MO) for 24 h at −55 °C and at 0.050 mBar vacuum. After freeze drying, dried microcapsules were stored at 4 °C for further experiments, and before use microcapsules were hydrated in peptone water and upon reconstitution the FD microcapsules rapidly regained their original spherical shape. Viable counts of non-encapsulated *L. acidophilus* NRRL-B 4495 were determined by a pour plate method using MRS agar after serial dilutions in peptone water. The plates were incubated anaerobically at 37 °C for 72 h, and colony forming units were estimated.

For enumeration of encapsulated bacteria, 10 g of FD microcapsules was diluted with peptone water and incubated at room temperature for 30 min for hydration. Microcapsules in peptone were then homogenized at 11,000 rpm for 5 min to release the bacteria. Samples of 1 ml from released bacteria were diluted to an appropriate dilution and plated by the pour plate technique using MRS agar. Colonies were counted after 72 h of incubation at 37 °C. The yield of encapsulation was determined as follows (Equation 1);

$$\text{Encapsulation yield } (\%) = (N_1/N_0) \times 100\%, \quad (1)$$

where N_1 is the total viable count of strains after microencapsulation and N_0 is the total viable count of strains before microencapsulation.

Viability under in vitro *gastric juice and bile salt solution*

Simulated gastric juice (SGJ) was prepared according to the method described by Guo et al. (2009) with some modifications. Saline solution was prepared, and the pH was adjusted to the 3.0 with 0.1N HCl and was sterilized at 121 °C for 15 min. Pepsin solution was prepared, sterilized by 0.22 μm filter and mixed with the saline at a final concentration of 3.0 g/l.

One gram of FD free or encapsulated bacteria were transferred into 9.0 g of SGJ and incubated at 37 °C under orbital shaking at 160 rpm for 3 h. After the incubation, samples were removed, and viable bacteria were enumerated. The control experiments were conducted by non-encapsulated bacteria as well.

The bile tolerance was examined according to the method described by Bao et al. (2010). One gram of free or encapsulated *L. acidophilus* NRRL-B 4495 were inoculated into 9.0 g of prepared bile salt solution (MRS medium supplemented with 0.6% ox-bile) and incubated at 37 °C under orbital shaking at 160 rpm for 24 h. After the incubation, samples were removed, viable bacteria were enumerated, and survival rate (%) was calculated. The control experiments were conducted by non-encapsulated bacteria as well. Survival rate (%) was calculated according to the following equation:

$$\text{Survival rate } \% = (\log \text{ CFU } N_1 / \log \text{ CFU } N_0) \times 100\%, \quad (2)$$

where N_1 is the total viable count of strains after treatment by gastric juice and N_0 is the total viable count of strains before treatment.

Release of encapsulated bacteria into in vitro *intestinal juice*

Simulated intestinal juice was prepared by supplementing PBS with trypsin. pH of PBS was adjusted to the 8.0 with 0.5 M NaOH and sterilized at 121 °C for 15 min. The trypsin solution was prepared at a final concentration of 1 g/l, then the filter was sterilized by 0.45 μm filter and added to the sterile PBS solution (Guo et al., 2009).

One gram of FD free or encapsulated cells were transferred into the 9.0 g of simulated intestinal juice and incubated at 37 °C under orbital shaking at 160 rpm for 24 h. After the incubation, samples were taken from the supernatant, and viable bacteria released in SIJ were enumerated. Survival rate (%) was calculated according to Equation (2). The control experiments were conducted by non-encapsulated bacteria as well.

Storage stability test

In order to examine the storage stability of the free and microencapsulated bacteria, both free and the microparticles were stored at 4 °C. The number of viable cell counts was determined weekly for 12 weeks.

Morpohology by microscopy examination and diameter distribution

The morphology of FD microcapsules was examined by scanning electron microscopy. FD microcapsules were placed on strips of double side carbon tape attached to aluminium SEM stubs, and photographs were taken at under low vacuum using an electron acceleration voltage of 10.0 kV with SEM (Quanta 250, FEI).

Diameter analysis was conducted by dispersing 1.0 g of microcapsule with 2–3 ml of deionized water, and 0.10 ml of microcapsule slurry was placed on a glass microscope slide with a cover slide. Microscopic pictures were taken using an Olympus CX31 Microscope, fitted with an Olympus DP25 Camera, and diameter analysis was done with software (Olympus DP2-BSW). The size distribution was measured in terms of the SPAN value, expressed in Equation (3) (Hundre et al., 2015):

$$\text{SPAN} = (D(0.9) - D(0.1))/D(0.5), \quad (3)$$

where $D(0.9)$, $D(0.1)$ and $D(0.5)$ are the diameters where the given percentage of particles are smaller than the stated size.

Moisture content and water activity measurements

Water activity of the microcapsules was determined using a Hygrolab C1 water activity meter (Hygrolab C1, Rotronic, Bassersdorf, Switzerland) (Dianawati et al., 2012). The moisture content of the microcapsules was determined gravimetrically by oven-drying at 105 °C for 24 h to reach weight equilibrium (Rajam et al., 2012). The mean MC was estimated by the following equation:

$$\text{MC } (\%) = [(W_0 - W_1)/W_0] \times 100, \quad (4)$$

where W_0 is the weight of the microcapsules before oven-drying and W_1 is the weight of fully dried microcapsules after oven-drying.

Colour measurement

Konica Minolta colorimeter (Model CR 410, Tokyo, Japan) was used for this experiment. The CIE Lab system was defined in rectangular coordinates (L^*, a^*, b^*), where L^* represents lightness, a^* represents red-green and b^* represents yellow-blue.

Swelling properties

The swelling degree of the FD bacteria-loaded WPI-pullulan microcapsules was characterized by suspending 10 FD microcapsules in denionized water/SGJ/SIJ overnight at 37 °C. After incubation, deionized water was removed from the medium carefully, and then the microcapsules were removed. Filter papers were used to absorb excessive water on the surface of the microcapsules which were then weighed (Klemmer et al., 2011).

The swelling degree (SD) values of FD WPI-pullulan microcapsules were calculated using the following equation:

$$\text{SD } (\%) = [(W_s - W_d)/W_d] * 100, \quad (5)$$

where W_s is the weight of the swollen microcapsules and W_d is the weight of FD microcapsules.

Statistical analysis

All the experiments were conducted in a triplicate ($n = 3$), and the results were expressed as mean ± standard deviation. Mean and standard deviation values were calculated from data obtained with triplicate trials. Data analysis was carried out using Minitab 14.0 software (Minitab Inc., State College, PA). Significance of differences between formulations was performed by one-way analysis of variance (ANOVA) followed by Tukey's test.

Results and discussion

Effect of freeze drying on cell viability

Results revealed that microencapsulation efficiency of 93.50% was achieved with WP/pullulan microcapsules. High-microencapsulation efficiency can be contributed to the non-toxicity of polymer blend and mild conditions of encapsulation process. Similarly, high-encapsulation efficiency values have been observed by other researchers (Doherty et al., 2011; Gebara et al., 2013; Rosas-Flores et al., 2013). However, methods and probiotic strains used in these studies are variable. The effect of freeze drying on both free and encapsulated cells is shown in Table 1. Combination of whey protein with pullulan resulted to a protective effect on survival of probiotic cells after freeze drying. Results from experiments showed that initial count of 9.41 log CFU/g has been reduced to 8.51 log CFU/g after freeze drying process for encapsulated bacteria. Microencapsulation resulted in the survival of probiotic bacteria with 90.42% survival rate. On the other hand, the log reduction of 1.48 which is equal to 84.39% survival rate was observed here in free cells. It seems that free cells of *L. acidophilus* used in this study are also resistant to freeze drying process. But further studies showed that FD free bacteria were more labile to simulated gastrointestinal conditions than encapsulated ones.

Table 1. Effect of freeze drying on survival of encapsulated *L. acidophilus* NRRL-B 4495.

	Initial cell number (log CFU/g)	Survived cell number (log CFU/g)	Survival rate (%)
Free bacteria	9.24 ± 0.18	7.76 ± 0.54	84.39 ± 1.63[a]
WPI-pullulan microcapsules	9.41 ± 0.88	8.51 ± 0.65	90.42 ± 2.81[b]

Note: [a,b]Mean ± standard deviation ($n = 3$) with different superscript letters in the same column indicates significant differences ($p < 0.05$) among the studied samples.

Polysaccharides and disaccharides including lactose, mannitol, maltodextrin, maltose, lactulose and inulin were reported to play an important role in protecting the viability of cells in freeze drying process. The cryoprotective effect of sugars during freeze drying is attributed to their capacity to form glassy structure providing protection of sensitive protein functionality in the cell and their ability to replace water associated with polar head groups of membrane lipids (Leslie et al., 1995; Carvalho et al., 2004; Fatemeh et al., 2011). Reid et al. (2005) reported an increase in survival rate of probiotic bacteria in whey protein microcapsules by supplemented sucrose and lactose during FD. Dianawati et al. (2013) used various protein–sugar combinations in order to observe the protective effect against freezing and freeze drying. Varying survival rates up to 97.4% were obtained, and as a result, the importance of combination of proteins with sugars in microencapsulation of probiotic bacteria was revealed.

Survival to *in vitro* gastric juice and bile salt solution

Figure 1 shows the viable counts of free and encapsulated *L. acidophilus* NRRL-B 4495 cells in FD, wet and free forms in both SGJ for 3 h and bile salt for 24 h. The results indicated that in SGJ, free bacteria showed a sharp decrease from 7.91 to 6.97 log CFU/g after 1 h, whereas in the presence of pullulan, detected viable cell numbers were 8.32 log CFU/g after 1 h declining from the initial count of 8.35 log CFU/g. Additionally in every sampling time, WPI-pullulan microcapsules obtained significantly higher survived cell numbers when compared to free cells. After 3 h exposure in gastric pH, free cells declined to 5.87 log CFU/g which is equal to 2.04 log cycles reduction in viability. This result suggested that free *L. acidophilus* NRRL-B 4495 was unable to show desired health effect that ingestion of free cells would result in reduced viability as previously stated. On the other hand, in WPI-pullulan microcapsule samples, about 1.12 log reduction in viable cell numbers was observed at the end of

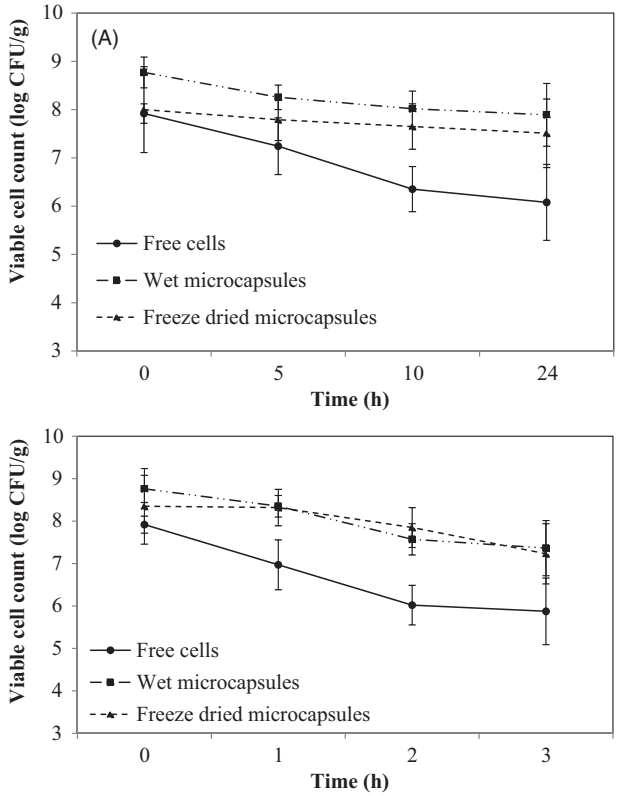

Figure 1. Viable counts of free and microencapsulated *L. acidophilus* NRRL-B 4495 in wet and FD forms under *in vitro* (A) acid conditions at 37 °C at pH 2.0 for 3 h, (B) bile salt at 37 °C for 24 h. Note: Values shown are mean ± standard deviations ($n = 3$).

Table 2. Viable cell numbers of wet and FD microencapsulated *L. acidophilus* NRRL-B 4495 under *in vitro* acid conditions at 37 °C at pH 2.0.

	Initial cell number (log CFU/g)	Survived cell number (log CFU/g)	Survival rate (%)
Free bacteria	7.91 ± 0.20	5.87 ± 0.17	74.19 ± 2.12[a]
FD WPI-pullulan microcapsules	8.35 ± 0.89	7.23 ± 0.29	86.58 ± 2.01[b]
Wet WPI-pullulan microcapsules	8.76 ± 0.32	7.36 ± 0.55	84.06 ± 1.78[b]

Note: [a,b]Mean ± standard deviation ($n = 3$) with different superscript letters in the column indicate significant differences ($p < 0.05$) among the studied samples.

exposure and obtained a viable number 7.23 log CFU/g. Table 2 also compares the survival rates of encapsulated *L. acidophilus* NRRL-B 4495 in free, wet and FD forms in SGJ. Microencapsulated FD cells showed a better survival when compared to wet ones as wet ones showed reduction of viable cell numbers from 8.76 to 7.36 log CFU/g which is equal to the net viable cell loss of 1.40 log CFU/g. Higher viability reduction in wet microcapsules was an expected results, because easier diffusion of acid through the pores of wet surface compared to dried ones has been previously reported by some authors (Fang et al., 2011; Nag, 2011).

Bile in small intestinal tract of the hosts has an important role as a surfactant and helps emulsify fats (Blackberg et al., 1981). The actual physiological concentration of human bile in duodenum is in the range of 0.3 to 0.5% (Vinderola and Reinheimer, 2003), and bile salts will be released once fatty meal is present. This natural phenomenon could be harmful for probiotic bacteria

Table 3. Viable cell numbers of wet and FD microencapsulated *L. acidophilus* NRRL-B 4495 under *in vitro* bile salt after 24h.

	Initial cell number (log CFU/g)	Survived cell number (log CFU/g)	Survival rate (%)
Free bacteria	7.92 ± 0.15	6.07 ± 0.57	76.76 ± 1.65[a]
FD WPI-pullulan microcapsules	8.00 ± 0.18	7.51 ± 0.35	93.87 ± 0.51[c]
Wet WPI-pullulan microcapsules	8.77 ± 0.21	7.89 ± 0.22	89.98 ± 1.21[b]

Note: [a–c]Mean ± standard deviation ($n = 3$) with different superscript letters in the same column indicate significant differences ($p < 0.05$) among the studied samples.

as their membrane composition comprises lipids and fatty acids (Liong and Shah, 2005). After incubation in bile salt solution, significant difference ($p < 0.05$) in survival rates and numbers between free and encapsulated cells was observed. As seen in Figure 1, the reduction in cell viability for free cells was 0.68 log CFU/g with a viable count of 7.24 log CFU/g after 5 h of incubation in bile salt. In presence of pullulan, encapsulated cells showed 3% increase in survival rates after 5 h of incubation. After incubation in bile solution for 10 h, the final viable cell numbers were 6.35 and 7.65 log CFU/g having the survival rates of 84.02 and 95.63% for free and WPI-pullulan encapsulated cells, respectively. It was calculated that free cells of *L. acidophilus* NRRL-B 4495 showed a net reduction of 1.84 log CFU/g with a survival rate of 76.76% after 24 h exposure to bile solution. In contrast, microencapsulation in the presence of pullulan, probiotic cells obtained significantly higher ($p < 0.05$) survival rate of 93.87%.

In comparison between the encapsulated cells, the viability of probiotic cells in wet microcapsules declined to 7.89 log CFU/g from a initial count of 8.77 log CFU/g. Freeze drying of microcapsules resulted in decrease in viable cell counts from 8.0 to 7.51 log CFU/g after 24 h of incubation in bile, in which survival rate was about 4% higher than that of wet microcapsules (Table 3). It seems that the action of bile salts on encapsulated bacteria was less pronounced for FD ones. Our results suggest that the shrinkage of microcapsules as a result of freeze drying causes reduction in the pore sizes and provide more difficult diffusion of both bile salts and SGJ into microcapsule core.

Microencapsulation into WPI-pullulan capsules could ensure greater survival in the gastric environment that cell number was high enough to meet the minimum probiotic theurophatic level required for beneficial effects. Albertini et al. (2010) studied incorporation of xanthan gum (XG) and cellulose acetate phthalate (CAP) into alginate for encapsulation of *L. acidophilus* LA14 and *Bifidobacterium lactis* BI07, and reported 7% increase in survival rate in SGJ by incorporating 0.5% XG to matrix. In another study, Dianawati and Shah (2011) incorporated mannitol in encapsulation system. After incubation in SGJ solution, encapsulated bacteria exhibited 0.106 log decrease in the presence of mannitol, whereas 1.47 log decrease was obtained in the absence of mannitol. However, after 8 h incubation in bile salt supplemented MRS broth, mannitol incorporation did not show any difference in viable cell numbers. Additionally, Lee at al. (2004) showed that microencapsulation with chitosan-Ca alginate improved the survival of probiotic *L. bulgaricus* cells in SGJ in FD forms. On the other hand, no change in viable numbers was calculated when cells were encapsulated in denatured WPI-sodium alginate. Oliveira et al. (2007) also encapsulated *Bifidobacterium lactis* (BI-01) and *L. acidophilus* (LAC 4) using the combination of casein and pectin and further dried by the use of spouted-bed technology. In this study, capsulated cells interestingly showed more sensitivity to low-pH values. Rajam

Table 4. Viable cell numbers of wet and FD microencapsulated *L. acidophilus* NRRL-B 4495 under *in vitro* simulated intestinal juice conditions at 37 °C at pH 8.0.

	Initial cell number (log CFU/g)	Released cell number (log CFU/g)	Release/ survival rate (%)
Free bacteria	8.47 ± 0.29	8.25 ± 0.10	97.40 ± 2.14^{c}
FD WPI-pullulan microcapsules	8.21 ± 0.16	7.41 ± 0.13	90.19 ± 1.58^{a}
Wet WPI-pullulan microcapsules	8.66 ± 0.05	8.22 ± 0.52	94.98 ± 0.67^{b}

Note: $^{a–c}$Mean ± standard deviation ($n = 3$) with different superscript letters in the same column indicate significant differences ($p < 0.05$) among the studied samples.

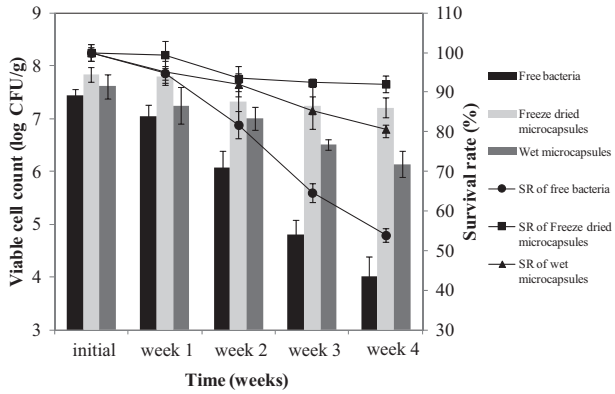

Figure 2. Viable counts of free and microencapsulated *L. acidophilus* NRRL-B 4495 4495 in wet and FD forms under storage at 4 °C for 12 weeks. Note: Values shown are mean ± standard deviations ($n = 3$).

et al. (2012) employed native/denatured WPI with sodium alginate for microencapsulation of *Lactobacillus plantarum* MTCC 5422. After 4 h incubation in MRS broth supplemented with 2% bile salt, no significant ($p > 0.05$) improvement in survival numbers was calculated in the case of encapsulation in native WPI-sodium alginate matrix. Hernández-Rodríguez et al. (2014) entrapped probiotic cells in WPI: k-carrageenan complex coacervates. As the ratio of protein to polysaccharide increased, nearly 3.5 log CFU/g increase in survived cell numbers was calculated after exposure to gastric acid and bile salts.

Release in *in vitro* intestinal juice

The release rates of WPI, WPI-pullulan encapsulated bacteria and release of free bacteria in simulated intestinal juice were revealed in Table 4. For all of the capsule designs, release of bacterial cells was shown to have high-release rates. Additionally, survival of FD free cells with 97% survival rate here suggests that once released, probiotic bacteria will be able to survive the simulated intestinal juice conditions. These results demonstrated that in FD forms, WPI-pullulan microcapsules were not as successful as wet ones for intestinal delivery of probiotic *L. acidophilus* as WPI capsules, but about 90% of WPI-pullulan encapsulated bacteria were released into the environment from FD microcapsules. Lower release rates for pullulan containing microcapsules can be explained by diffusion limitation due to denser capsule structure and non-digestion of pullulan in SIJ. However, pullulan is able to be completely fermented by intestinal microbiota (Wolf et al., 2003; Peters et al., 2011) therefore in *in-vivo* studies, it is expected to show higher release rates. Release of encapsulated bacteria mostly depends on the type of the polysaccharide incorporated into the wall matrix. Cheow et al. (2014) incorporated locust bean gum (LBG) and xanthan gum (XG) in wall matrix for microencapsulation of *L. rhamnosus*. The release of encapsulated bacteria was evaluated by subsequent exposure microcapsules from SGJ to SIJ for 4 h. Final released bacteria were calculated as 5.5 and 4.7 log CFU/mg for LBG and XG, respectively. In another study, alginate-pectinate complex was selected for the microencapsulation, and it is observed that after 2.25 h, all the entrapped cells were released from the FD capsules. Gebara et al. (2013) used pectin-whey protein microparticles and observed a reduction of 354 units for free cells. On the other hand, native or denatured whey protein-pectin encapsulated cell decreased about 1.59 and 1.67 log units, respectively. Another consideration for release of encapsulated cells was the strength of interactions between polymer matrix and encapsulated cells. Sanders and Klaenhammer (2001) revealed in their study that interactions between denatured whey protein and different strains of probiotic *Lactobacillus* cannot be attributed to electrostatic forces. Atomic force microscopy (AFM) results showed that the main driving forces governed for interactions might be specific forces including Van der Waals, hydrophobic and steric interactions. Moreover, the adhesiveness of microorganisms through denatured whey proteins was explained by the surface composition such as the presence of exopolysaccharides (EPS) and glycosylated proteins which depend on the type of bacterial strain (Sanders and Klaenhammer, 2001; Phillips, 2009). In this research, our findings showed that the release rate of encapsulated cells were high which can be attributed to lack of strong interactions.

Storage stability test

In addition to survival through gastrointestinal conditions, microencapsulation should also ensure the viability during the storage. In this study, FD microcapsules and FD free bacterial cells were stored at refrigeration temperature for 12 weeks. It is clear that microencapsulation of probiotic bacteria cells leads to a higher survival compared to free bacteria cells under same conditions. For free bacteria, gradual decrease in viable cell numbers thus in survival rate was observed, and at 12 weeks of storage, no viable cells were detected (Figure 2). Protective effect of microencapsulation was clearly observed during storage stability test. However, this protective effect is more pronounced for FD microcapsules; WPI-pullulan microencapsulated bacteria obtained 92.1, 89.41 and 88.45% survival rate with the viable cell counts of 7.21, 7.00 and 6.91 log CFU/g after 4, 8 and 12 weeks of storage, respectively. Furthermore, it was observed that during storage, no significant changes in surface composition of both wet and dry microcapsules occurred but dry microcapsules increased in size due to hydration. However, this increase was insignificant ($p > 0.05$), and no crack formation on the surface or distinct changes were observed showing that encapsulated cells did not proliferate and increase in number during storage.

In dried microcapsules, the diffusion of oxygen can be limited during the storage and detrimental effects of oxygen therefore become less pronounced (Selmer-Olsen et al., 1999). In case of WPI-pullulan microcapsules, incorporation of pullulan provided smoother surface with smaller pore sizes. Therefore, possible explanation for the protective effect of WPI-pullulan micro-encapsulation could be its enhanced physical barrier function hence leading to a higher survival rates.

Morphological studies and diameter distribution

Particle size analyzes were conducted to determine the average size distribution of WPI-pullulan microcapsules. In industrial production, low-capsule sizes are required since high-capsule sizes above 100 µm create sandy and gritty undesirable textural property in the food product in which they are used (Annan

et al., 2008; Vivek, 2013). Table 5 represents the particle size parameters for the WPI-pullulan microcapsules. The microcapsules with mean particle size of $D(0.5) = 73.34\,\mu m$ with estimated pore sizes of less than 200 nm were produced, whereas *L. acidophilus* are approximately 0.6–0.9 µm in width and 1.5–6.0 µm in length (Prasad, 2013). $D(0.9)$ value represented that 90% of the total sample volume have microcapsules with diameters below 98.07 µm. Moreover, small SPAN value of 0.75 demonstrated that WPI-pullulan polymer blend achieved microcapsules with narrow particle distribution in size. The frequency distribution of the FD microcapsules ranged from 15.75 to 175.87 µm. When the microcapsule sizes were examined in detail, high-particle size frequencies were calculated between 45 and 95 µm, while below 10% distribution was calculated for large-sized microcapsules (>100 µm) with the lowest distribution of 1% calculated in the 165–170 -µm interval. Moreover, the frequency distribution also showed that it is skewed more towards the

Table 5. Particle size parameters of WPI-pullulan microcapsules.

SPAN value	$D(0.1)$	$D(0.5)$	$D(0.9)$
0.75 ± 0.03	$44.54 \pm 3.63\,\mu m$	$73.34 \pm 2.02\,\mu m$	$98.07 \pm 2.98\,\mu m$

Note: Values shown are mean ± standard deviations ($n = 3$).

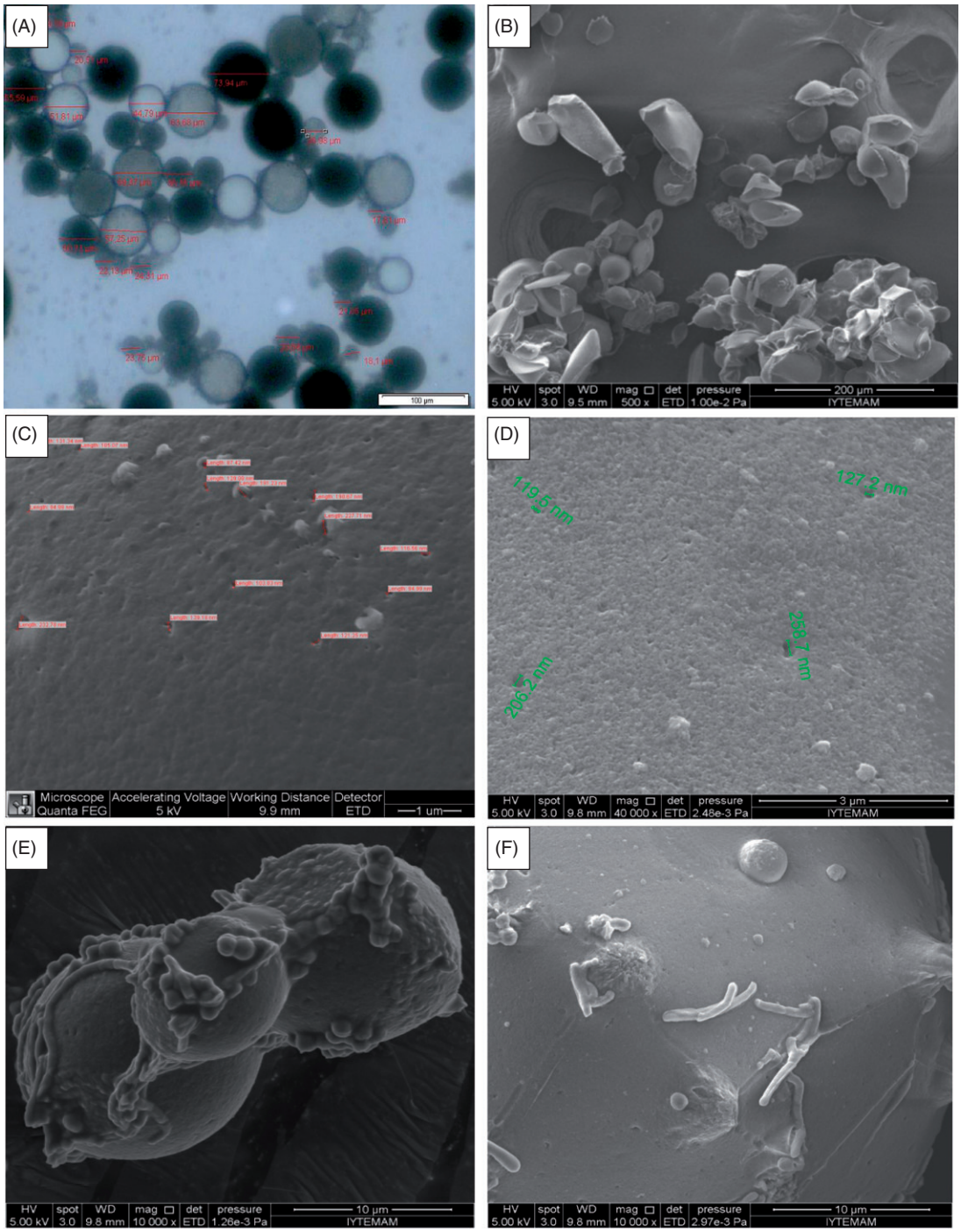

Figure 3. Optical microscopy images of WPI-pullulan microcapsules (A) and scanning electron microscopy (SEM) images of FD WPI-pullulan microcapsules without bacteria (B), surface of FD WPI-pullulan (C), surface of FD WPI microcapsules (D), FD WPI-pullulan microcapsules with bacteria (E), FD surface of WPI-pullulan microcapsules with bacteria (F).

Table 6. Moisture content, swelling degree and water activity values of FD microencapsulated *L. acidophilus* NRRL B-4495.

	WPI-pullulan microcapsules
Moisture content (%)	6.14 ± 1.16
Swelling degree in dH_2O (%)	198.20 ± 5.23
Swelling degree in SGJ (%)	69.11 ± 4.33
Swelling degree in SIJ (%)	103.23 ± 6.89
Water activity (A_w)	0.062 ± 0.04
Colour	
L^*	90.88 ± 0.34
a^*	−1.52 ± 0.01
b^*	3.51 ± 0.02

Note: Values shown are mean ± standard deviations ($n = 3$).

smaller diameter values side relative to the most frequently occurring value.

Optical microscopy images of microcapsules and scanning electron microscopy (SEM) images of FD microcapsules were shown in Figure 3. Wet WPI-pullulan microcapsules were spherical in shape shown in light microscopy images (Figure 3A). It was also shown in Figure 3(B) that due to freeze drying, slight disruption of the spherical structure occurred, and the agglomerated FD microcapsules were observed. It can be explained by the possible existence of residual oil droplets on the formed microcapsules (Su et al., 2011). In microencapsulation technology, porosity of wall material is an important factor for the preservation and survival of encapsulated bioactive materials (Allan-Wojtas et al., 2008). As it is seen from SEM images, inclusion of pullulan in wall material created denser and smoother surface, and provided reduced pore sizes with relatively broad distributions (Figure 3C), whereas it is observed that the largest pore sizes are presented in the microcapsules obtained in WPI without pullulan (Figure 3D). Detailed examination of the microcapsule surface at higher magnifications revealed that probiotic bacteria were both buried and attached to the surface of WPI-pullulan microcapsules (Figure 3E, F).

As expected small pore sizes of WPI-pullulan microcapsules appeared to provide better barrier properties against bile and acid diffusion when compared to WPI microcapsules. Evaluation of SEM results with *in vitro* gastrointestinal tests suggests that addition of pullulan provided protective effect against diffusion, since it provides smoother surface and smaller pore sizes; WPI-pullulan microcapsules therefore can effectively be used in frozen foods.

Moisture content, water activity, colour and swelling degree

The average moisture content, water activity, swelling degree and colour values of FD probiotic microcapsules are given in Table 6. *A*w (water activity) value of FD microcapsules was also found to be within desirable limits (0.28–0.65) for the survival of probiotic bacteria (Kosanke et al., 1992) and colour values (L^*, a^*, b^*) of the formed microcapsules showed that these microcapsules had white colour. On the other hand, maximum level moisture content of dry products required for prolonged storage was stated to be at or below 4%. Higher moisture content above 10% is not desired as caking usually occurs in powders with high-moisture contents and creates problems during handling (Masters, 1979; Desmond et al., 2002). In this research, freeze drying of microcapsules resulted in the formation of powders with moisture content and water activity of 6.14 and 0.06%, respectively. It can be seen that moisture content of FD samples are higher than the recommended levels but a direct conclusion based on moisture content is not possible, since the moisture distribution in the microcapsules can be inhomogeneous with respect to the different constituents of the samples (Linders et al., 1997). Moreover, higher moisture content levels of FD probiotic microcapsules with high-survival rates during storage were reported by other authors (Reid et al., 2005; Chávez and Ledeboer, 2007; Kim et al., 2008; Gbassi et al., 2009). Additionally, during storage stability tests, no adverse effects such as cake formation and microbial growth such as moulds were not visually observed. The swelling degree is the rehydration capacity of the dried microcapsules when exposed to water. WPI-pullulan microcapsules retained a significant fraction of water within its structure having 198.2% swelling degree which indicates that few or no ionic linkages between whey protein and pullulan since lower swelling degree is generally attributed to the higher degree of cross-linking within polymer matrix.

Conclusion

Due to ability to form glass structures and ability to interact with cell membrane components, sugars are generally preferred as cryoprotective agents during freeze drying. In the present study, survival rate of *L. acidophilus* was enhanced by employing pullulan in protein encapsulation matrix. WPI-pullulan microencapsulation retained more than 90% of viable cells after freeze drying as compared to free and WPI encapsulated ones. Although WPI-pullulan microcapsules need to be further investigated for characterization of the encapsulation system and need to be tested in *in-vivo* conditions, obtained microcapsules efficiently improved the resistance to *in-vitro* gastrointestinal conditions in FD forms. Moreover, analysis demonstrated that obtained polymer matrix could release enough viable cells recommended by the International Dairy Federation (10^7 CFU/g), viable cells for potential health benefits. SEM analysis of microcapsules indicated that the presence of pullulan in wall matrix provided the formation of smoother surfaced microcapsules that could limit diffusion of harsh acidic conditions and cell leakage of bacteria. In conclusion, use of pullulan offers an effective cryoprotective behaviour when combined with whey protein in microencapsulation process.

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Declaration of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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