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Whey Protein-Pullulan (WP/Pullulan) Polymer Blend for Preservation of Viability of *Lactobacillus acidophilus*

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In this study, whey protein isolate-pullulan (WP/pullulan) microspheres were developed to entrap the probiotic *Lactobacillus acidophilus* NRRL-B 4495 by spray-drying technique. Microcapsules were analyzed for physicochemical characteristics including morphology, particle size, moisture content, water activity, dissolution time, and color properties. Results revealed that microcapsules were spherical in shape and obtained particle sizes between 5 and 160 µm, with an average size of around 50 µm. Blending pullulan with WP provided enhanced survival of probiotic bacteria during spray drying with a final viable cell number of 8.81 log CFU/g of microcapsule. Encapsulated probiotics were also found to have significantly ($p \leq 0.05$) higher survived cell numbers compared to free probiotics under detrimental gastrointestinal conditions. Moreover, dissolution analysis suggested that protein-polysaccharide powdered microcapsules showed pH-sensitive dissolution properties in simulated gastric juice and simulated intestinal juice.

Keywords Polymer blend; Probiotic; Pullulan; Spray drying; Whey protein

INTRODUCTION

The intestinal microbial community has an essential role in maintaining human health, since microbial balance provides beneficial fermentation products and increases the bioavailability of essential minerals.[1,2] The relationship between food consumption and intestinal health is becoming increasingly important. Probiotic products occupy one of the largest areas within the functional food group. After many studies conducted on probiotics, the International Dairy Federation (IDF) has stated that, in the colon, a concentration of 10^7 live cells per gram or ml of the product should exist in order to exert expected benefits.[3]

Many nutraceutical and functional food components, including vitamins, probiotics, prebiotics, omega-3 fatty acids, plant extracts, antimicrobials, antioxidants, flavors, colors, and minerals, have been encapsulated in appropriate edible delivery systems in order to provide efficient survival under gastrointestinal conditions and to improve their viabilities during shelf-life.[4–7] Spray drying is one of the oldest encapsulation methods adapted to many industrial areas to make powders and capture bioactive components. The spray-drying technique has been widely applied for microencapsulation of flavors, fish oil, probiotics, and vitamins.[8] The protective effects of microencapsulation on probiotics have been extensively investigated.[9–12] However, high heat treatment during the spray-drying method may cause cellular injuries and death for temperature-sensitive probiotic microorganisms.[13,14] Therefore one of the most important points in spray drying is selecting a suitable matrix to avoid high heat loss and to meet the specific requirements for the objective of the current microencapsulation process.

Due to their amphiphilic nature and good emulsifying characteristics, proteins offer suitable properties required for microencapsulation of probiotics. Native or denatured whey protein is the most widely studied protein for the microencapsulation of probiotics. During heat treatment, whey protein undergoes denaturation and some structural changes.[15,16] Studies have revealed that, due to unfolding by preheating treatment of whey protein, improved functional and encapsulation properties such as gelation and adsorption at the interface have been reported.[17–20] Whey protein has also proved to be an effective encapsulating agent because of its ability to form microcapsules easily under mild conditions using different techniques.[21–24] For instance, Lee and Rosenberg[22] used whey protein as an encapsulation agent for the protection of the drug theophylline. In the study, it was concluded that, with retention efficiency of higher than 74%, theophylline was not affected by encapsulation conditions.

On the other hand, studies revealed that polysaccharides show good solubility in water and posses low viscosity at high concentrations, compared to proteins. Excellent oxygen and moisture barrier properties of some polysaccharides also provide good protection for the encapsulated bioactive materials.[25–27] Pullulan is an extracellular polysaccharide

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produced by *Aureobasidium pullulans*.[28] This polysaccharide is especially used as a coating material in the food and pharmaceutical industries due to its non-toxic, water-soluble, colorless, tasteless, odorless, and heat-stable characteristics.[29] Ravi and Vats[30] developed pullulan nanoparticles for the delivery of human immunodeficiency virus (HIV) protease inhibitor Laponivar. According to the results of this study, it was observed that high microencapsulation efficiency values were obtained. Moreover, nanoencapsuated Laponivar was observed to have higher metabolic protection and bioavailability when compared to free Laponiva. In another study, the effect of nanoencapsulation of adriamycin in pullulan capsules was studied to overcome the drug resistance of cancer cells. The results showed significant improvement due to encapsulation and revealed that adriamycin was released in significant amounts with encapsulation in pullulan-based nanocapsules.[31] Pullulan was also used to encapsulate *B. animalis* Bb12 using the electrospinning technique.[32] Milk and phosphate-buffered saline (PBS) were used as dissolving media instead of water. Pullulan capsules were compared with capsules made of whey protein concentrate and it was found that pullulan alone was not as effective as whey protein concentrate in the stabilization of *B. animalis* Bb12 cells.

Therefore, the present study aimed at researching the protective effect of denatured whey protein and pullulan combination, enhancing the viability of probiotic *L. acidophilus* NRRL-B 4495cells following spray drying and and evaluating the survivability of encapsulated cells under *in vitro* gastrointestinal conditions.

MATERIALS AND METHODS

Materials

A commercial strain of *L. acidophilus* NRRL-B 4495 was received from the USDA-ARS Culture Collection. Ox-bile and de Man, Rogosa and Sharpe (MRS) media were purchased from Fluka (Buchs, Switzerland). Pullulan was kindly provided by Food Ingredients Hayashibara Shoji, Inc. (Okayama, Japan). Trypsin (from bovine) was purchased from Merck (Darmstadt, Germany) and pepsin (from porcine stomach mucosa) was purchased from Sigma (St. Louis, MO, USA). Commercial whey protein isolate (WP), Bipro, was obtained from Davisco International Inc. (LeSueur, Eden Prairie, MN, USA).

Methods

Bacterial Strain and Culture Preparations

L. 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 the same conditions for 12 h. The cells were harvested by centrifugation at 15.000 rpm for 10 min at 4°C from 20 ml of a 12 h culture in the late log phase. The supernatant was discarded, and the cells were re-suspended in 100 ml of WP/pullulan blend solution, obtaining a cell load of about 9.0–10.0 log CFU ml^{-1}.

Formation of WP/Pullulan Wall Material

Whey protein isolate-pullulan microcapsules were prepared according to the method of Wood[33] with some modifications. Briefly, whey protein isolate (WP, 9% w/v) was dispersed by mixing the protein powder in sterile distilled water at ambient temperature. Protein solution was then 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.

Pullulans were dissolved in distilled water at ambient temperature and stirred for approximately 3 h using a magnetic stirrer to ensure proper dissolution. In order to form a WP/pullulan polymer blend as a wall material, the pullulan solution was then sterilized using a 0.45 μm filter and mixed with the denatured WP solution (9.0%, w/v) at a final concentration of 2.0% (w/v).

Preparation of Microcapsules

WP alone (control) or a WP/pullulan polymer blend containing *L. acidophilus* NRRL B-4495 with the initial cell load of approximately between 9.0–10.0 log CFU/g was microencapsulated by spray drying carried out using a laboratory spray dryer (Büchi Mini Spray dryer B-290, Büchi Labortechnik AG, UK). In this study, all conditions were fixed, including aspiration rate of 70%, flow rate of the peristaltic pump 15 ml/min, and inlet and outlet air temperatures of 150°C and 50°C. The powders were collected in a single-cyclone separator. The microencapsulation of bacteria was performed in triplicate. The resultant spray-dried bacteria were stored separately in 5 g quantities in sealed sterile glass bottles at 4°C.

Bacterial Enumeration

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. The enumeration of microencapsulated bacteria in microcapsules were calculated according to the methods proposed by Hernández-Carranza and López-Malo.[34] Briefly, 10 g of spray-dried control or WP/pullulan microcapsules were suspended in peptone water and constantly stirred at 890 rpm for 5 min for release. Samples of 1 ml of the peptone water 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 viable cell number was expressed as

CFU per gram of microcapsule (CFU/g) and the yield was determined following Eq. (1):

$$\text{Survival rate } (\%) = 100 \times (N/N_0) \quad (1)$$

where N_0 was the number of bacteria before drying, and N was the number of bacteria in the spray-dried powder.

In-Vitro Gastrointestinal Survival

Simulated gastric juice (SGJ) was prepared according to the method described by Guo and Wang,[35] with some modifications. Saline solution (0.85%) pH was adjusted to 2.0 using 0.1 N HCl and sterilized by autoclaving at 121°C for 15 min. Pepsin was dissolved in sterile deionized water and filtered through 0.22 μm sterile membrane filter, then suspended in sterile saline to a final concentration of 3.0 g/L. To prepare bile salt solution, MRS media was supplemented with 0.6% ox-bile.[36] 1.0 ml of free or 1.0 g of microencapsulated *L. acidophilus* NRRL-B 4495 were inoculated into 9.0 ml of sterile SGJ/bile salt solution and incubated at 37°C under orbital shaking at 160 rpm for 3 h/24 h. After the incubation, samples were removed from solutions and survival rate (%) was calculated by Eq. (2).

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

N_1 = the total viable count of strains after treatment by gastric juice

N_0 = the total viable count of strains before treatment

Release in Simulated Intestinal Juice

Saline solution (0.85%) pH was adjust to 8.0 using 0.5 M NaOH and sterilized by autoclaving at 121°C for 15 min. Trypsin was dissolved in sterile deionized water and filtered through a 0.45 μm sterile membrane filter, then suspended in sterile saline solution to a final concentration of 1 g/L.[35] 1.0 ml of free or 1.0 g of microencapsulated bacteria were transferred into 9.0 ml of simulated intestinal juice and incubated at 37°C under orbital shaking at 160 rpm for 24 h. After incubation, samples were taken from supernatant and viable bacteria released in SIJ were enumerated. Released rate (%) was calculated according to Eq. (3). The control experiments were conducted by non-encapsulated bacteria, as well.

$$\text{Release rate } \% = (\log \text{ CFU } N_1/\log \text{CFU } N_0) \times 100\% \quad (3)$$

N_1 = the total viable count of strains released in SIJ

N_0 = the total viable count of powdered strains added to SIJ

Characterization of Microcapsules

Morphology of Microcapsules. The morphology of both WP and WP/pullulan microcapsules formed by spray drying was examined by both scanning electron microscopy (SEM) and environmental scanning electron microscopy (ESEM). For SEM images, microcapsules were placed on strips of double-sided carbon tape attached to aluminum SEM stubs and photographs were taken at under low vacuum using an electron acceleration voltage of 10.0 kv with SEM (Quanta 250, FEI). Optical microscope images were taken using an Olympus CX31 Microscope, fitted with an Olympus DP25 Camera and diameter analysis was done with software (Olympus DP2-BSW).

Particle Size and Color Measurement. 0.1 g of microcapsules 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). A Konica Minolta colorimeter (Model CR 410, Tokyo, Japan) was used for color measurements of microcapsules. 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.

Moisture Content and Water Activity. Water activity of the microcapsules was determined using a Hygrolab C1 water activity meter (Hygrolab C1, Rotronic, Bassersdorf, Switzerland).[37] The moisture content of the microcapsules was determined gravimetrically by oven-drying at 105°C for 24 h to reach weight equilibrium.[38] The mean MC was estimated by Eq. (2):

$$MC(\%) = \left[\left(W_{wet} - W_{dry}\right)/W_{wet}\right] * 100 \quad (2)$$

where W_{wet} is the weight of the wet microcapsules and W_{dry} is the weight of fully dry microcapsules.

Storage Stability. In order to examine the storage stability, both free bacteria and spray-dried WP and WP/pullulan microcapsules after production were placed at 4°C in glass bottles with compressed N_2 for four weeks. The number of viable cell counts was determined weekly for four weeks.

Statistical Analysis

Experiments were performed with three different batches of drying, and each batch was tested in triplicate. Results were expressed as means ± standard deviation. Data analysis was carried out using Minitab 14.0 software (Minitab Inc., State College, PA). Significance of differences between formulations was performed by analysis of variance (ANOVA) test followed by Tukey's test (95% confidence interval).

RESULTS AND DISCUSSION

In Vitro Gastrointestinal Survival

Figure 1 shows the time course of the survival of free and spray-dried cells of *L. acidophilus* NRRL B-4495 during

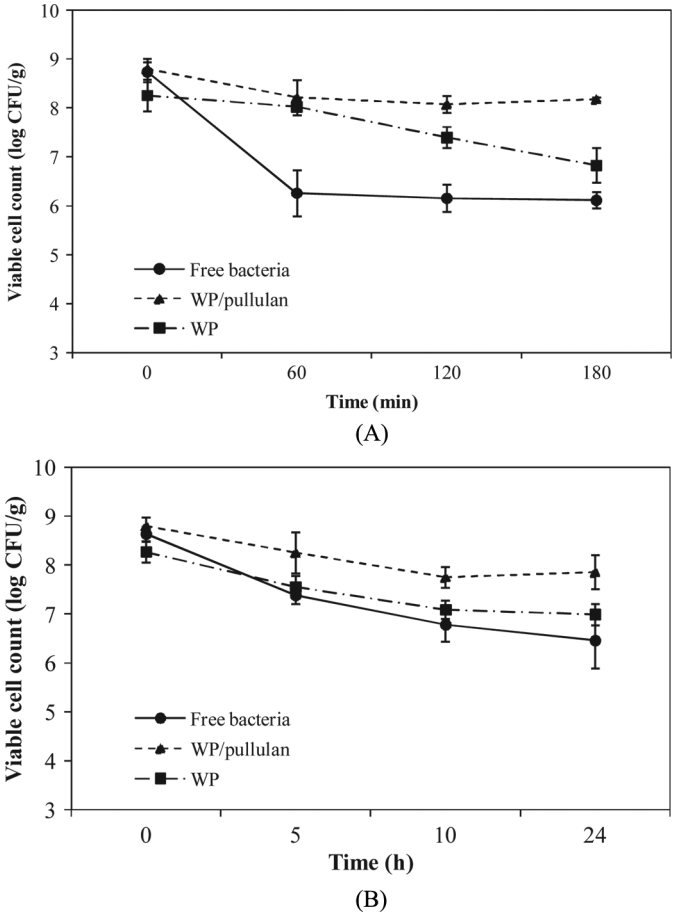

FIG. 1. Survived cell counts of (●) free and spray-dried *Lactobacillus acidophilus* NRRL B-4495 with (■) WP and (▲) WP/pullulan after exposure to (A) simulated gastric juice at 37°C at pH 3.0; and (B) bile salt solution at 37°C. Values shown are means ± standard deviations (n = 3).

exposure to SGJ and bile salt solution. It was found that the survived cell numbers of free and microencapsulated cells showed significant ($p \leq 0.05$) difference. After 60 min of incubation in SGJ, the viability of free bacteria declined to 6.24 log CFU/g and finally declined to 6.12 log CFU/g from the initial count of 8.74 log CFU/g after 180 min of incubation (Fig. 1A). In contrast, survival of encapsulated bacteria was higher than that of the free cells; survival rate of probiotic bacteria increased 23% by encapsulation in WP/pullulan polymer blend. When compared with initial cell number, spray drying in the presence of a WP/pullulan polymer blend decreased only 4% in viable cell numbers and reached to 8.18 log CFU/g from initial count of 8.80 log CFU/g after 180 min. WP microcapsules were also subjected to SGJ where significant difference ($p \leq 0.05$) was calculated in viable cell numbers compared to WP/pullulan. Overall, the microencapsulation process resulted in an increased viable cell numbers in the presence of pullulan, where survival rates were found as 82.68% and 93.01% for WP and WP/pullulan microcapsules, respectively.

The survived cell numbers of microencapsulated cells in bile salt solution were significantly ($p \leq 0.05$) higher than free cells throughout the exposure. Between 2 and 3 h of incubation, viable cell numbers of encapsulated bacteria remained unchanged while free bacteria decreased as the exposure time increased (Fig. 1B). A significant difference ($p \leq 0.05$) was found in survival rates of probiotic bacteria after 24 h incubation in bile salt solution. Encapsulated cells of *L. acidophilus* NRRL B-4495 in WP/pullulan microcapsules showed a decrease of 11.0%, representing 1.0 log cycles corresponding to 7.86 log CFU/g from an initial count of 8.80 log CFU/g, while WP showed a decrease of 25%, representing 1.27 log cycles corresponding to 6.99 log CFU/g from an initial count of 8.27 log CFU/g after 24 h. On the other hand, free *L. acidophilus* NRRL B-4495 cells decreased to 1.86 log CFU/g after 10 h of incubation and finally obtained a cell count of 6.46 log CFU/g from an initial count of 8.64 log CFU/g after 24 h.

The higher survival rates in encapsulated bacteria can be attributed to the buffering effect of whey protein[39,40] and dissolution behavior of WP/pullulan microcapsules. In order to have more information, disintegrations of microcapsules following exposure to SGJ and bile salt solution were visualized under SEM, respectively (Fig. 2). As seen, microcapsules of WP/pullulan blend showed smoother and more compact structure compared to WP and were not fully dissolved, only partially destroyed due to pepsin and bile action. The compact and smooth structure of WP/pullulan microcapsules can be attributed to the formation of possible intermolecular ionic interaction and hydrogen bonds between pullulan and WP.[41–43] As a result, the formation of compact structure by the interaction between pullulan and whey protein provided slow rehydration, thus slow degradation of WP/pullulan microcapsules. The compact structure provided more difficult diffusion of both bile salts and simulated gastric juice into the microcapsule core, therefore it prevented the extensive degradation of formed microcapsules (Fig. 2D and Fig. 2E) compared to WP (Fig. 2A and Fig. 2B) and protected cells from the harsh environmental conditions that they are exposed to. As a result, the solutions obtained in this present study showed that spray drying with WP/pullulan could offer good protection for *L. acidophilus* NRRL B-4495 against gastric juice and bile salts. Moreover, the viable cell number faster exposure to SGJ and bile salt achieved the required minimum level (7 log CFU/g). High survival rates with the incorporation of pullulan have been observed earlier by Leonardi and Gerbault.[44] The blending of pullulan with Eudragit® S100 was observed to have a protective effect on the release of risedronate in SGJ. With some polymer blend formulations, similar results have also been reported. For instance, Lapsiri and Bhandari[45] obtained improved surviving cell numbers of spray-dried *L. plantarum* when maltodextrin or trehalose

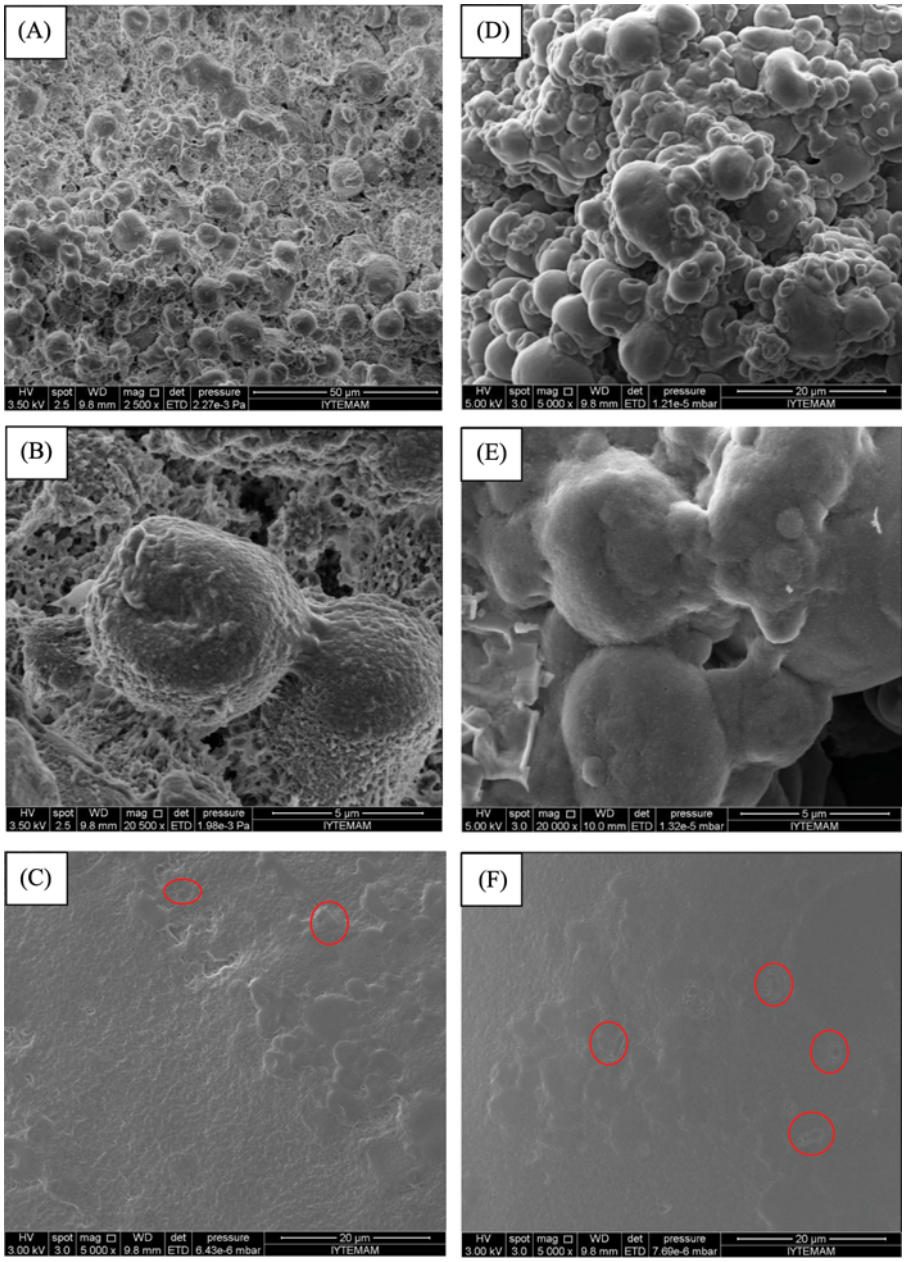

FIG. 2. Scanning electron microscopy images of WP microcapsules after (A) SGJ, (B) bile salt, (C) SIJ exposure; and scanning electron microscopy images WP/pullulan microcapsules after (D) SGJ, (E) bile salt, (F) SIJ exposure.

was added to the wall material. In another study, *L. paracasei* NFBC 338 was spray dried in the presence of gum acacia and encapsulated bacteria were more resistant to simulated gastric stress than control cells, resulting in 6 log reduction in the viability of control while encapsulated cells showed 4.0 log reduction after 120 min exposure at 37°C.[46] Fritzen-Freire and Prudêncio[11] evaluated the effect of inulin and oligofructose on the survival of *B. Bifidum* BB-12 under stress conditions. It was found that spray drying in the presence of inulin and oligofructose-enriched inulin promoted the survival of encapsulated bacteria when exposed to bile salt solution (5.0 g/L). Significant ($p \leq 0.05$) improvements in resistance to simulated gastrointestinal conditions have also been observed by Favaro-Trindade and Grosso,[47] O'riordan and Andrews,[48] and Páez and Lavari,[9] depending on the spray-drying matrix. Improved bile resistance of spray-dried *L. acidophilus* A9 was reported by Páez and Lavari,[9] but viable cell number was not high enough (3.99 log CFU/ml) for further beneficial effects.

In Vitro Gastrointestinal Release

In emulsion encapsulation and extrusion technique, probiotic bacteria are entrapped within hydrogels which generally remain water-insoluble. In the spray-drying technique, microcapsules prepared from polysaccharides and proteins are water-soluble and, therefore, during incubation under *in vitro* gastrointestinal conditions, encapsulated cells are released. For this reason, release rates of WP and WP/pullulan encapsulated cells following exposure to SGJ over 24 h was observed. Results in Fig. 3 demonstrated that released behavior of WP/pullulan microcapsules appeared similar to WP microcapsules and cell counts of released bacteria increased with exposure time. In the first 30 min of incubation, 87.05% and 85.27% of encapsulated cells liberated reaching viable cell numbers of 7.26 log CFU/g and 7.24 log CFU/g for WP and WP/pullulan microcapsules, respectively. After 3 h exposure in SIJ, 8.23 log CFU/g and 7.93 log CFU/g viable cells with release rates of 98.57% and 93.4% were liberated from WP and WP/pullulan microcapsules while complete cell liberation was performed following 5 h of incubation in SIJ. Overall, results support our hypothesis that the incorporation of pullulan to the whey protein improved the cell survival characteristics, retarding the diffusion and erosion rate of SGJ and bile salt while allowing the release of encapsulated cells in SIJ. A similar release behavior of dried probiotic has been reported by Morelli.[49] He observed that encapsulated bacteria were not released into SGJ in significant numbers, while high numbers of viable cells were enumerated after passing to the intestinal region with pH > 6. The image obtained using SEM in Fig. 2C and Fig. 2F shows the disintegration of WP and WP/pullulan microcapsules at pH 8.0 in SIJ. It can be concluded that both WP and WP/pullulan microcapsules lost their structural integrity and dissolved completely to liberate encapsulated cells in SIJ solution. Moreover, encapsulated bacteria within both dissolved microcapsules shown in circles can be seen (Figs. 2C and 2F).

Storage Stability

Figure 4 describes the changes in viable cell numbers of microencapsulated cells during refrigerated storage for four weeks. Results showed that free cells had the highest loss of viability compared to encapsulated cells. Viable cell numbers of free cells decreased to 6.48 log CFU/g representing 76.14% survival rate at the end of storage. On the other hand, the differences in survived cell numbers observed in this study indicated that pullulan incorporation significantly ($p \leq 0.05$) enhanced the protection of *L. acidophilus* NRRL B-4495 throughout storage; cell numbers using WP/pullulan polymer blend as a microcapsule wall material decreased from 8.60 log CFU/g to 8.48 log CFU/g after two weeks and further decreased to 7.9 log CFU/g, which represents a 92.55% survival rate at the end of storage. Meanwhile, under the same storage conditions, WP microcapsules exhibited 14.46% loss in survival rate representing 1.22 log CFU/g decrease in viable cell numbers from initial count of 8.43 log CFU/g at the end of storage.

The stability of dried probiotic is mainly related to the water activity and moisture content of final product. For

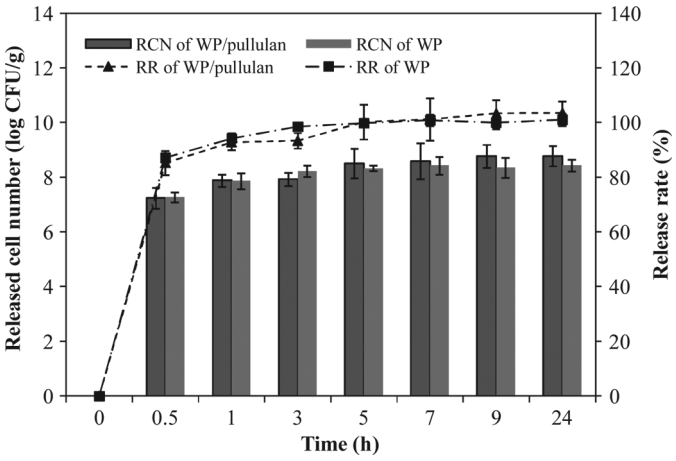

FIG. 3. Released cell counts and release rates of spray-dried *Lactobacillus acidophilus* NRRL B-4495 with (■) WP and (▲) WP/pullulan in SIJ (pH 8.0) after 3 h exposure to SGJ (pH 3.0). Values shown are means ± standard deviations (n = 3).

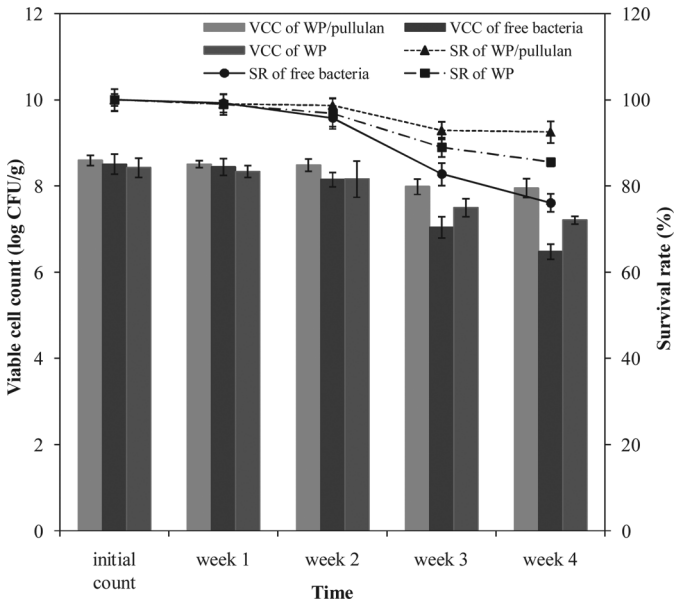

FIG. 4. Viable cell counts of (●) free and spray-dried *Lactobacillus acidophilus* NRRL B-4495 in (■) WP and (▲) WP/pullulan under storage at 4°C for four weeks. Values shown are means ± standard deviations (n = 3).

example, Teixeira and Castro[50] reported that high A_w value accelerated mortality during storage of *L. delbruekii*. Also, the presence of some simple sugars and polysaccharides has been reported to increase storage stability of dried probiotics by replacing water molecules in cell membranes due to hydroxyl groups of carbohydrates, thus stabilizing the structure of proteins by hydrogen bonding with polar groups of proteins.[51] In another study, *B. bifidum* BB-12 encapsulated in the presence of gum arabic, gelatin, and pectin using a spray-drying method[13] and nearly 2.0 log CFU/g, 2 .0 log CFU/g and 1.5 log CFU/g decrease in viable cell numbers was calculated for gum arabic, gelatin, and pectin, respectively, after one month of storage at 5°C. Goderska and Czarnecki[52] used different types of starch (N-tack Hylon VII, N-lok) to encapsulate *L. acidophilus* DSM 20079. Initial counts of 1.24×10^{10} CFU/g decreased to 4.42×10^{7} CFU/g after 10 days and further storage did not decrease the viable cell numbers below 10^{7} CFU/g in the presence of N-tack starch. On the other hand, Rodríguez-Huezo and Durán-Lugo[53] found that none of the polymer blend combinations (whey protein-maltodextrin-mesquite gum, gum arabic- mesquite gum, gum arabic-mesquite gum-maltodextrin) succeeded in reaching the desired level of viable cell numbers (7 log CFU/g) after five weeks of storage.

Characterization of Microcapsules

Physicochemical Characterization and Diameter Distribution

Moisture content and water activity affect the stabilities of the microcapsules throughout storage; higher moisture contents and water activities have been noted to increase oxygen permeability of wall materials and cause a higher decline in surviving viable numbers of microorganisms during storage.[54,55] Incorporation of pullulan to the spray-drying matrix markedly influenced the moisture content and A_w values of spray-dried microcapsules. WP microcapsules revealed moisture content and water activity (A_w) of 3.34 and 0.37, respectively (Table 1). On the other hand, moisture content and water activity (A_w) values for WP/pullulan microcapsules were found to be 4.21 and 0.41, respectively. As seen, spray-dried WP/pullulan microcapsules obtained higher water activity value compared to WP ones, which can be due to the increase in the number of polar sites with the presence of pullulan, since the presence of sugars result in an increased moisture content of a final product.[56,57] On the other hand, different results in moisture content and A_w due to the incorporation of sugars were stated by Chávez and Ledeboer.[58] Soy protein isolate was blended with lactose, maltodextrin, and sucrose. Calculated moisture content values were 0.354, 0.367, and 0.207, respectively, when compared to WP having moisture content value of 0.213.

Moisture content values for spray-dried probiotic microcapsules vary depending on the used polymer type, but it has been reported by various researchers that about 4.0% moisture content is an ideal level for prolonged survival of probiotic bacteria during storage.[59] In our

TABLE 1
Physicochemical characterization of spray-dried WP and WP/pullulan microcapsules loaded with *L. acidophilus* NRRL B-4495

Physicochemical characteristics	WP/pullulan microcapsules	WP microcapsules
Moisture content (%)	4.21 ± 0.06^{A}	3.34 ± 0.034^{B}
Water activity (A_w)	0.41 ± 0.13^{A}	0.37 ± 0.21^{B}
Color		
L*	91.21 ± 1.96^{A}	92.17 ± 0.67^{A}
a*	-1.12 ± 0.08^{A}	-1.08 ± 0.05^{A}
b*	4.89 ± 0.29^{A}	4.21 ± 0.10^{A}

$^{A\text{-}B}$Means ± standard deviation with different superscript letters in the same column indicate significant differences ($P < 0.05$) among the studied samples.

L* indicates lightness, a* is the position between green (negative values) and red/magenta (positive values), and b* is the position between blue (negative values) and yellow (positive values).

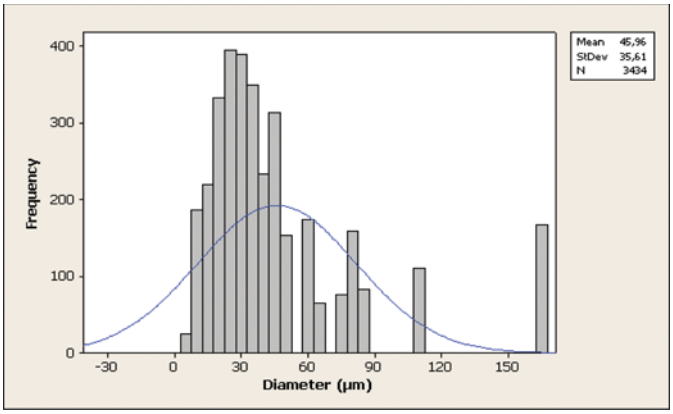

FIG. 5. Frequency distribution of diameters of the spray-dried WP/pullulan microcapsules.

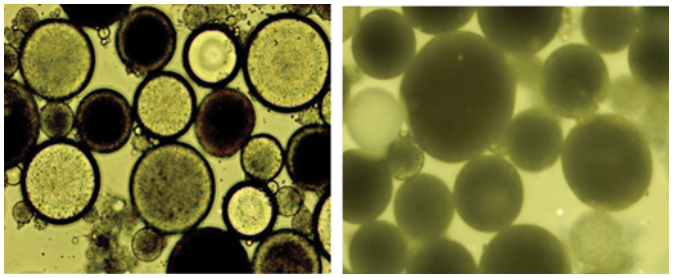

FIG. 6. Optical microscopy images of WP/pullulan microcapsules.

conducted research, formed WP/pullulan microcapsules obtained acceptable moisture and water activity levels for spray-dried powders.

Generally, during spray drying of proteins in the presence of reducing sugars, a Maillard reaction takes place causing shades in color tending to red and yellow.[10] Color values of the formed microcapsules showed that these microcapsules had white color and no significant change ($p > 0.05$) occurred due to high heat treatment in color when compared to WP microcapsules. This feature might allow the application of these spray-dried microcapsules in dairy-based products without any visual disadvantage.

Microcapsules with a high frequency of large-sized particles have been reported to affect the textural and sensory acceptability of added food products.[4] Mean diameters smaller than 100 μm are generally preferred to avoid the formation of undesirable sensory and textural problems.[60,61] The particle size distribution of WP/pullulan spray-dried microcapsules loaded with *L. acidophilus* NRRL B-4495 is illustrated in Fig. 5. The frequency distribution

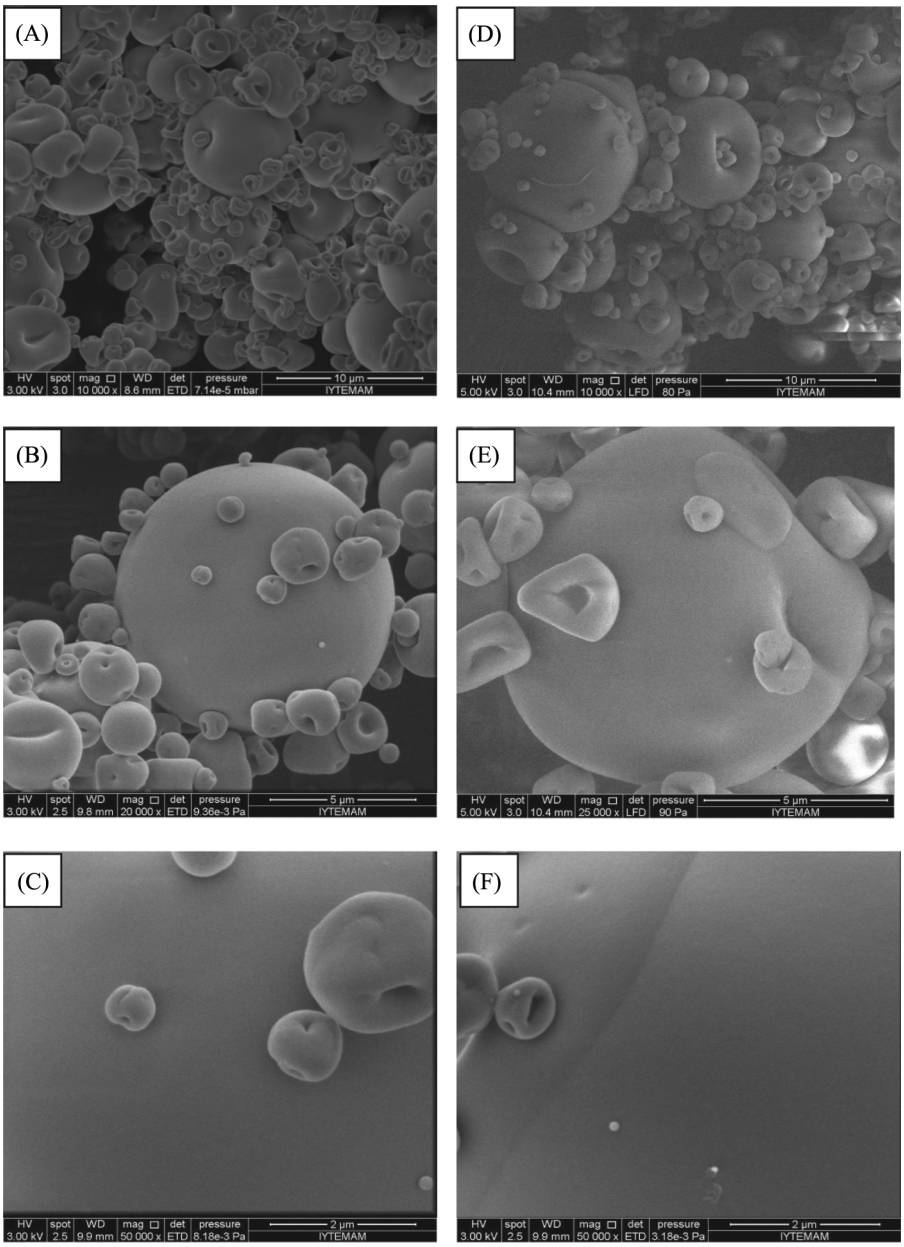

FIG. 7. Scanning electron microscopy images of WP/pullulan microcapsules after production under (A) 10,000×, (B) 20,000×, and (C) 50,000× magnifications; and after storage for four weeks under (D) 10,000×, (E) 20,000×, (F) 50,000× magnifications.

curve of microcapsule sizes revealed that microcapsule sizes ranged from approximately 5 to 160 μm, with an average size of around 50 μm, and within 3434 samples only 350 samples exhibited mean diameters above 100 μm. De Castro-Cislaghi and Silva[10] reported mean microcapsule size around 11 μm with whey. Soukoulis and Behboudi-Jobbehdar[62] used whey protein concentrate with maltodextrin to encapsulate *L. acidophilus* NCIMB 701748 and reported microcapsules having mean diameter of 10.96 μm. Crittenden and Weerakkody[63] microencapsulated *B.infantis* Bb-02 and obtained small microcapsules having particle sizes below 20 μm. However, it seems that, when compared with the literature, mean particle size of WP/pullulan microcapsules was obtained in slightly larger form. This could be due to the type, solid content ratio, and viscosity of polymer used in feed solution[64,65] and spray-drying conditions including inlet and outlet temperature and moisture content of the final product.[66–68]

Morphological Analysis

Morphological characterization of spray-dried WP/pullulan microcapsules containing *L. acidophilus* NRRL B-4495 was carried out by optical and scanning electron microscopy. Optical microscopy and SEM images showed that relatively spherical microcapsules with homogenous surface were obtained by the polymer blend (Figs. 6 and 7). The probiotic cells did not appear on the surface of microcapsules, indicating that they were trapped within the microcapsule. All SEM images revealed that WP/pullulan microcapsules exhibited a smooth and dense surface structure, which provided protection for the encapsulated cells. This smooth structure limited the diffusion of acidic gastrointestinal solutions and thus restricted the release of encapsulated bacteria. Additionally, microcapsules varied in size and some shape irregularities were observed in microcapsules. Similar shaped irregularities, especially in the presence of polysaccharides called a ''flat ball effect'' in spray-dried microcapsules, have been reported by some researchers.[10,53,69] Figure 7 also shows the morphological changes of spray-dried microcapsules after four weeks of storage under SEM. It was observed that, during storage, no significant changes in surface morphology of microcapsules occurred. Additionally, no crack formation on the surface or distinct changes were observed, showing that encapsulated cells did not proliferate and increase in number during storage. This could be attributed to the formation of a smooth structure within the microcapsule; free volume for the bacteria needed to proliferate inside the microcapsule was insufficient.[70]

CONCLUSION

These results demonstrated that WP/pullulan microcapsules could be a promising wall material for microencapsulation of *L. acidophilus* NRRL-B 4495. Low dissolution of spray-dried probiotic microcapsules in gastric acid and bile salt, resulted in encapsulated cells that showed higher survival rates when compared to free ones. An *in vitro* release test was carried out and release data clearly showed that WP/pullulan microcapsules released most of the *L. acidophilus* NRRL-B 4495 cells in the first 30 min. Moreover, results also showed that encapsulated cells survived at the minimum desired level recommended by the International Dairy Federation (7 log CFU/g) at low pH values, in contrast to free cells. SEM analysis of the microcapsules indicated that the presence of pullulan in wall material provided the formation of smoother surfaced microcapsules that could limit diffusion of harsh acidic conditions and cell leakage of bacteria. WP/pullulan microcapsules need to be further investigated for characterization of the encapsulation system and need to be tested under *in vivo* conditions; obtained microcapsules efficiently improved the resistance to *in vitro* gastrointestinal conditions, resulting in viable cells for potential health benefits.

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