Protection of *Lactobacillus acidophilus* NRRL-B 4495 under *in vitro* gastrointestinal conditions with whey protein/pullulan microcapsules

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In this research, whey protein/pullulan (WP/pullulan) microcapsules were developed in order to assess its protective effect on the viability of *Lactobacillus acidophilus* NRRL-B 4495 under *in vitro* gastrointestinal conditions. Results demonstrated that WP/pullulan microencapsulated cells exhibited significantly (*p < 0.05*) higher survival than free cells after 3 h incubation in simulated gastric solution. Moreover WP/pullulan microcapsules were found to release over 70% of encapsulated *L. acidophilus* NRRL-B 4495 cells within 1 h. The effect of encapsulation during refrigerated storage was also studied. Free bacteria exhibited 3.96 log reduction while WP/pullulan encapsulated bacteria showed 1.64 log reduction after 4 weeks of storage.

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**Key words:** Encapsulation; Pullulan; Whey protein; *Lactobacillus acidophilus*; Probiotic

Viability losses during storage and gastrointestinal transit due to detrimental conditions such as harsh acidic environment, oxygen stress and enzymatic reactions reduce the functionality of probiotics to exert health benefits (1,2). Thus, microencapsulation of probiotics is considered as an effective approach for their efficient survival under gastrointestinal conditions and to improve the viability during shelf life to maintain their health promoting effects. Additionally, mechanical protection of probiotic cells during incorporation process into food product is another advantage of microencapsulation (3,4). Despite wide applications of several microencapsulation materials for probiotics, it is a challenge to produce microcapsules for preservation of desired bacterial culture with high viability which does not cause non desirable texture in the final product. Proteins and polysaccharides are widely used materials for the microencapsulation of bioactive ingredients (5–8). 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 (9,10).

Pullulan is an extracellular polysaccharide produced by *Aureobasidium pullulans*. It is especially used as a coating material in the food industry, paper industry and pharmaceutical area because of its non-toxic, water-soluble, colorless, tasteless, odorless and heat stable characteristics. Many different industrial wastes such as potato peel, grape skin and olive oil wastes found to be used as a carbon source for the synthesis of pullulan (11,12). More recently, combination of proteins, especially whey proteins, with polysaccharides has been studied for microencapsulation of bioactive substances. Whey protein and pullulan mixtures have been studied to form edible films to reduce moisture loss and increase the shelf life in food applications (13,14).

The present study aimed at microencapsulation of probiotic *Lactobacillus acidophilus* NRRL-B 4495 within whey protein/pullulan biopolymer blend and evaluating the survivability of encapsulated cells under *in vitro* gastrointestinal conditions. We also attempted to examine protective effect of polymer matrix during storage.

**MATERIALS AND METHODS**

**Materials** Commercial strain of *L. acidophilus* NRRL-B 4495 was obtained from the ARS Culture Collection (NRRL, USA). Ox-bile and de Man, Rogosa and Sharpe (MRS) media were purchased from Fluka (Buchs, Switzerland). Trypsin (from bovine) was purchased from Merck (Darmstadt, Germany) and pepsin (from porcine stomach mucosa) from Sigma (St. Louis, MO, USA). Sunflower oil was obtained from a local store. Pullulan was a gift from Hayastabaka Co. (Japan). Whey protein isolate (WP) was obtained from BiPro, Danisco. All other chemicals were obtained from Sigma.

**Bacterial strain and culture preparations** *L. acidophilus* NRRL-B 4495 cells were inoculated into 5 ml of MRS broth in 0.1% ratio 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 rpm for 10 min at 4°C from 20 ml of a 12 h culture at the initial stationary phase. The supernatant was decanted, and the cells were re-suspended in 100 ml of pullulan-WP solution obtaining a cell load of about 9.0 log CFU ml⁻¹.

**Formation of WP/pullulan wall matrix** Whey protein isolate-pullulan microcapsules were prepared according to the method of Wood (15) with some modifications. Briefly, whey protein isolate (WP, 9% w/w) 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 ice bath.

Pullulan was 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 WP/pullulan polymer blend as a wall material, pullulan solution was then sterilized by 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

Microcapsules were prepared by emulsification/cold gelation technique performed as described by Chen and Subirade [16] with some modifications. In the first step, primary water-in-oil emulsions (W1/O) were formed by emulsifying an inner aqueous phase (W1) made up by WP/ pullulan polymer blend containing bacteria into an oil phase (O) containing 1% Polycryllylic polyisocyanate (PCPy) as an emulsifier. W1/O emulsion was prepared by using an Ultra Turrax homogenizer (Ultra Turrax, model T25, IKA Labowtechnik, Staufen, Germany) at 3000 rpm for 15 min. This emulsion was then added with gentle mixing to CaCl2 solution (100 mM). After formation of microcapsules, this slurry was orbital 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 10000 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.

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 counted. For enumeration of microencapsulated bacteria in microcapsules, 10 g of microcapsules were suspended in peptone water. This peptone water containing microcapsules was homogenized at 11,000 rpm for 5 min. Under these conditions, microcapsules were broken and 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 anaerobic incubation at 37°C. Viable cell number was express as CFU per gram of microcapsule (CFU/g) and the efficiency was determined as following Eq. 1:

\[
\text{Encapsulation yield (}% = \frac{N_1}{N_0} \times 100
\]

where N is the total viable count of L. acidophilus after microencapsulation and N0 is the total viable count of L. acidophilus before microencapsulation.

Survival in simulated gastrointestinal conditions

Simulated gastric juice (SGJ) was prepared according to the method described by Guo et al. [17] 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 [18].

For resistance in simulated gastrointestinal conditions, 1.0 ml of free or 1.0 g of microencapsulated L. acidophilus NRRL-B 4495 cells 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:

\[
\text{Survival rate } \% = \frac{\log \text{CFU } N_1}{\log \text{CFU } N_0} \times 100
\]

where N1 is the number of viable cells released from microcapsules in SIJ and N0 is the number of viable cells added to SIJ.

Release into simulated intestinal juice

Saline solution (0.85%) pH was adjusted 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 0.45 μm sterile membrane filter, then suspended in sterile saline solution to a final concentration of 1 g/L [17]. 1.0 g of microencapsulated bacteria were transferred into the 9.0 ml 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 supernatant and viable bacteria released in SIJ were enumerated. Released rate (%) was calculated according to Eq. 3:

\[
\text{Release rate } \% = \frac{\log \text{CFU } N_1}{\log \text{CFU } N_0} \times 100
\]

where N1 is the number of viable cells released from microcapsules in SIJ and N0 is the number of viable cells in microcapsules added to SIJ.

Moisture content and water activity

Water activity of the microcapsules was determined using a Hygroball C1 water activity meter (Hygroball C1, Rotronic, Basersdorf, Switzerland) [19]. The moisture content of the microcapsules was determined gravimetrically by oven-drying at 105°C for 24 h to reach weight equilibrium [20]. The mean moisture content (MC) was estimated by the following Eq. 4:

\[
\text{MC (}% = \frac{W_{\text{final}} - W_{\text{dry}}}{W_{\text{final}}} \times 100
\]

where W0 is the weight of the wet microcapsules and W1 is the weight of the dry microcapsules.

Storage stability

In order to examine the storage stability, both free bacteria and the microcapsules were placed at 4°C in glass bottles for 4 weeks with compressed N2 for 4 weeks. The number of viable cell counts was determined weekly and all the samples were analyzed in triplicate.

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, USA). Significance of differences between formulations was performed by analysis of variance (ANOVA) test followed by Tukey’s test (95% confidence interval).

RESULTS AND DISCUSSION

Microencapsulation

Table 1 shows that existence of pullulan resulted in a bigger mean diameter size of microcapsules providing to a microcapsule size of 76.40 μm, whereas WP microcapsules obtained mean particle size of 65.12 μm. This can be attributed to the increased polymer concentration of wall matrix due to pullulan incorporation. By the increase in polymer concentration, the viscosity of the inner phase of the primary emulsion might be increased causing resistance to break into smaller droplets and resulting in larger microcapsule sizes [21-24]. Besides, increased particle mean diameter, results revealed that no significant (p > 0.05) difference was calculated between encapsulation efficiencies of pullulan containing and non pullulan containing microcapsules. Encapsulation efficiency is one of the most important parameter indicating the effect of encapsulation process and selected wall matrix. Cell loading achieved with WP/ pullulan was calculated to be 93.72%, while microencapsulation efficiency of 93.57% was achieved with WP microcapsules. The average count of L. acidophilus NRRL-B 4495 in WP/pullulan microcapsules was 93.3 ± 0.21 log CFU/g.

Survival under simulated gastric juice and simulated bile salt solution

One of the main objectives of microencapsulation is providing protection of probiotic cells during exposure to low pH gastric environment. Fig. 1A shows the number of survived cell counts of free and microencapsulated L. acidophilus NRRL B-4495 under simulated gastric juice. After an hour of incubation, L. acidophilus NRRL-B 4495 was reduced by 0.95 log units when added as free cells and followed nearly a linear reduction in cell numbers. At the end of incubation, viable cell numbers of free cells decreased to 6.67 log CFU/ml possessing the survival rate of 73.19%. On the other hand, microencapsulation into WP and WP/
pullulan microcapsules offered significant \( p \leq 0.05 \) protection throughout the incubation time. Moreover, results indicated that bacterial cells were better protected in the presence of pullulan in wall material after 3 h exposure to low pH; WP/pullulan microcapsules exhibited decreasing cell loss of 1.17 log CFU/g from initial cell count of 9.2 log CFU/g, while viable counts of WP encapsulated cells decreased about 1.88 log CFU/g from initial cell count of 9.44 log CFU/g. Microencapsulation into WP/pullulan microcapsules offered protection with survival rate of 87.18% compared to WP microcapsules possessing a survival rate of 80.04%.

The effect of the bile salt on the cell counts of free and microencapsulated \textit{L. acidophilus} NRRL B-4485 is presented in Fig. 1B. For free cells, initial viable count of 9.31 log CFU/ml was reduced to 7.75 log CFU/ml after 10 h and were further reduced to 7.10 log CFU/ml at the end of incubation. In contrast to free cells, after 10 h of incubation in bile salt solution, cell counts of WP and WP/pullulan microcapsules decreased to 7.71 log CFU/g and 8.52 log CFU/g from initial counts of 8.93 log CFU/g and 9.27 log CFU/g, respectively. Beyond 10 h of incubation, viable cells of encapsulated bacteria showed slight decreases.

This increased survival rates could be attributed by the restricted diffusion of SGJ and bile salts; formation of smoother and denser surface in microcapsules with the presence of pullulan limited the diffusion of harsh gastrointestinal fluids into microcapsules core, leading to protection of encapsulated cells. Moreover, it can be hypothesized that gel network formed in the presence of pullulan also reduced the degradation of whey protein by restricting the diffusion of pepsin (25).

There are various studies conducted to observe the effect of different polymers on probiotic survival when exposed to lethal conditions. Lotfpour et al. (26) incorporated polysaccharide psyllium in alginate to encapsulate probiotic bacteria of \textit{L. acidophilus} DMSZ20079 and concluded that increasing psyllium concentration resulted in an increase in viable cell numbers when microcapsules exposed to \textit{simulated} gastric acid. Nag et al. (27) combined sodium caseinate with gellan gum for the protection of \textit{Lactobacillus casei}. Formed microcapsules exhibited 3.1 log reduction when compared to control having 4.6 log reduction in viable cells. On the other hand, incorporation of any polysaccharide into protein wall matrix does not always provide barrier functions. For instance, Guerin et al. (28) incorporated pectin into whey protein wall matrix and used this polymer complex to encapsulate probiotic \textit{Bifidobacterium bifidum}. After exposure of formed microcapsules to simulated gastrointestinal conditions, they observed a significant reduction in

\[ \text{TABLE 2. Viable cell counts of } L.\text{ acidophilus NRRL-B 4495 microencapsulated with different pullulan concentrations under storage at 4}^\circ\text{C after 4 weeks.} \]

<table>
<thead>
<tr>
<th>WP-pullulan</th>
<th>Initial cell number (log CFU/g)</th>
<th>Survived cell number (log CFU/g)</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free bacteria</td>
<td>9.59 ± 0.17</td>
<td>5.55 ± 0.21</td>
<td>57.92 ± 0.23*</td>
</tr>
<tr>
<td>WP microcapsule</td>
<td>9.44 ± 0.12</td>
<td>7.91 ± 0.21</td>
<td>81.89 ± 0.53b</td>
</tr>
<tr>
<td>WP/pullulan microcapsule</td>
<td>9.51 ± 0.17</td>
<td>7.87 ± 0.31</td>
<td>82.80 ± 0.43b</td>
</tr>
</tbody>
</table>

\*Means ± standard deviation \((n = 3)\) with different superscript letters in the same column indicate significant differences \( p < 0.05 \) among the studied samples.
viable cell numbers. It was explained by using SEM images in details and concluded that having a more porous structure of the microcapsule, easier the penetration of acid and bile facilitates that causes a reduction in the entrapped cell number.

Release into simulated intestinal juice

The release characteristic of the developed WP/pullulan microcapsules was investigated since release of viable cells in the intestine is one of the main objectives of microencapsulation. For this assay, microcapsules were first exposed to SGJ at pH 3.0 for 3 h after which the same microcapsules were transferred into SIJ at pH 8.0 and incubated for further 24 h. The investigated intestinal release characteristic of the encapsulated cells is revealed in Fig. 2. Results indicated that cell counts of released bacteria increased with exposure time. In 1 h of incubation in SIJ, more than 70% of encapsulated bacteria were liberated from WP/pullulan microcapsules, and within 7 h release of encapsulated cells was completed reaching viable cell numbers of 9.41 log CFU/g. On the other hand, encapsulated cells in WP microcapsules were calculated to release at the rate of 88.75% within 1 h of exposure and after 3 h exposure in SIJ, release of all encapsulated bacteria appeared to occur with 9.11 log CFU/g viable cells at the end of 3 h of exposure to SIJ. Pullulan is a slowly digested carbohydrate by human gastrointestinal enzymes (25,29). This behavior of pullulan to intestinal enzymes should have stabilized the gel characteristics of WP/pullulan microcapsules in the intestine after exposure to SGJ and bile. Moreover, larger pore sized microcapsules were obtained by pullulan that was expected to limit the diffusion of SIJ solution. Since, large diameter size capsules have a lower surface area-to-volume ratio, thus these capsules usually would not undergo the same rate of capsule matrix degradation as the smaller-sized ones causing more prolonged release profile (30,31).

Storage stability

To evaluate the efficiency of addition of pullulan into microencapsulation wall material under refrigerated storage, free cells and WP/pullulan microcapsules were stored for 4 weeks. It was clear that microencapsulation of probiotic bacteria cells led to a higher survival compared to free bacteria cells under same conditions (Table 2). For free bacteria, gradual decrease in viable cell numbers thus low survival rate was observed. Initial cell counts of 9.59 log CFU/ml decreased to 5.55 log CFU/ml for free cells after 4 weeks of storage, while microencapsulation into WP/pullulan microcapsules afforded a decrease of viable cell numbers from 9.51 log CFU/g to 7.87 log CFU/g after 30 days of

FIG. 3. Frequency distribution of diameters of the WP/pullulan microcapsules.

FIG. 4. Scanning electron microscopy images of WP/pullulan microcapsules after production under (A) 3500× and (B) 10,000× magnifications and environmental scanning electron microscopy image of a surface (C) and cross-section (D) of WP/pullulan microcapsules. Scale bars represent 30, 10, 20 and 50 µm, respectively.
storage at 4°C. Moreover, after 30 days of storage, probiotic cells survived above desired therapeutic level (7 log CFU/g capsule). On the other hand, no significant difference \((p > 0.05)\) was calculated between survival rates of WP and WP/pullulan microcapsules. In contrast to our observation, various reports exposing positive effects of polysaccharide incorporation to capsule wall materials exist in the literature. Oliveira et al. \((32)\) used complex coacervation followed by the spouted-bed-drying method for microencapsulation of \(L.\) acidophilus \((LAC\) 4) in casein/pectin polymer complex. After storage for 30 days, no significant decrease in cell numbers was calculated; viable cells decreased from 9.16 log CFU/g to 8.92 log CFU/g. In another study, López-Rubio et al. \((33)\) used electrospinning technique to encapsulate \(Bifidobacterium\) strains in a protein \((\text{whey protein concentrate})\) and a carbohydrate \((\text{pullulan})\). After 120 days of storage, pullulan microcapsules exposed a significant drop of 4.0 log units \((p < 0.001)\) in viable cells of \(B.\) animalis Bb12 when compared to WPI encapsulated cells having decrease of 2 log units.

**Physicochemical characterization of microcapsules** Color values of the formed microcapsules in Table 3 reveal that these microcapsules have white color and can be applied dairy based food such as yogurt, cheese and whey based drinks. However, the color of the formed microcapsules may undergo some color changes when added to different food groups and this may be another topic of further research.

The moisture content and water activity \((A_w)\) of the microcapsules was found to be 82.36% and 0.95, respectively (Table 3). 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 higher decline in surviving viable numbers of microorganisms during storage \((34)\). Furthermore, low moisture content and water activity values in microcapsules contribute to improve physical and bulk properties \((35)\). In this work, WP/pullulan microcapsules exhibited high water activity and moisture content values that can create some problems for use in some food products for long time storage. For this reason, for the possible prolonged storage of encapsulated bacteria in functional foods, it was necessary to study the effect of drying to reduce the moisture content for practical uses.

Microcapsule size is an important parameter that both reflect the quality of added food product and protection of cells during gastrointestinal transit. In industrial production, low capsule sizes are required since high capsule sizes above 100 \(\mu\)m create sandy and gritty undesirable textural property in the food product in which they are used \((36,37)\). In this study, incorporation of pullulan was expected to create large particle sizes since by the increase in polymer concentration, the viscosity of the inner phase of the primary emulsion was increased, causing resistance to break into smaller droplets and resulted in larger capsule size \((21,22)\). Fig. 3 shows the frequency of diameter distribution of developed WP/pullulan microcapsules and results revealed that microcapsules obtained in this work had an average mean diameter of 76.40 \(\mu\)m and diameter distributions were between 30 and 172 \(\mu\)m. Besides large particle sizes above 100 \(\mu\)m, highest frequencies for the microcapsules were obtained between 55 and 95 \(\mu\)m. Moreover the frequency distribution of large sized microcapsules was lower than 10 and is skewed more towards the smaller diameter values side relative to the most frequently occurring value. In literature \((8)\), with small microcapsules as capsule sizes surface area to volume increases can further lead to reduced survival during gastrointestinal transit; smaller diameter in microcapsules generally results in decreased microencapsulation efficiency and protection against harsh conditions. As a result frequency distribution of microcapsules represents that by emulsification/cold gelation method, with WP/pullulan polymer blend formed microcapsules showed ideal size for food applications. Different attempts were made in order to encapsulate probiotic microorganism to provide survival during gastrointestinal storage in effective ways and microcapsules with varying mean diameters were obtained. For instance, Wang et al. \((30)\) used chickpea protein incorporated with \(\kappa\)-carrageenan/alginate to encapsulate \(Bifidobacterium\) \(\text{adolescentis}\) \((\text{ATCC 15703})\) cells using the emulsion technique. In the study, the presence of \(\kappa\)-carrageenan created larger particle sizes of about 838.5 \(\mu\)m when...
Microcapsule morphology Morphological structure and shape of microcapsules are illustrated by scanning electron microscopy (SEM) and environmental scanning electron microscopy (ESEM) (Fig. 4). It is observed in Fig. 4A that the formed microcapsules are spherical in shape with a smooth and homogeneous surface having small pores. From the images, it was confirmed that probiotic L. acidophilus NRRL B-4495 cells were effectively encapsulated and encapsulated cells are visible on the external surface of the microcapsules in Fig. 4B. The morphology from ESEM images (Fig. 4C,D) indicates that compact, homogeneous and smooth structure throughout the microcapsule was observed due to the formation of pululan thus providing better barrier properties to stop diffusion of acid and bile and release of encapsulated bacteria when compared to whey protein microcapsules. Similar results were noted by López-Rubio et al. (33) using electrosprinning technique. On the other hand, according to the investigation conducted by Gounga et al. (14), the incorporation of pululan in whey protein creates pinholes on the surfaces of probiotic edible films. Therefore, protein-pululan interactions need to be investigated.

Stability of formed WP/pulullan microspheres during exposure to simulated gastrointestinal conditions was visualized under light microscopy. From the appearances of eroded surfaces in Fig. 5, it can be considered that WP/pulullan microcapsules were partially destroyed due to pepsin action and cracks on surface of polymer layer were not observed in bile exposed ones. It may be due to limited penetration of artificial gastric solutions into the core was limited supporting the maintenance of shape and viable cell numbers above required levels. Light microscopy image after sequential exposure in SIJ provides information that after 24 h WP/ pulullan microcapsules were completely degraded and enabling the release of encapsulated cells. In conclusion, the diffusion of SIJ increased and encapsulated bacteria released in high numbers in a short time. Similar to our results, effective disintegration of microcapsules under simulated gastrointestinal conditions and release of encapsulated cells have been previously reported (39–41).

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