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**Abstract**

The aims of this study were to develop a milk protein-based probiotic delivery system using a modified rennet-induced gelation method and to determine how the skim milk powder concentration level and pH, which can affect the rennet-induced intra- and inter-molecular association of milk proteins, affect the physicochemical properties of the probiotic delivery systems, such as the particle size, size distribution, encapsulation efficiency, and viability of probiotics in simulated gastrointestinal tract. To prepare a milk protein-based delivery system, skim milk powder was used as a source of milk proteins with various concentration levels from 3% to 10% (w/w) and rennet was added to skim milk solutions followed by adjustment of pH from 5.4 or 6.2. *L. rhamnosus* GG was used as a probiotic culture. In confocal laser scanning microscopic images, globular particles with a size ranging from 10 μm to 20 μm were observed, indicating that milk protein-based probiotic delivery systems were successfully created. When the skim milk powder concentration was increased from 3% to 10% (w/w), the size of the delivery system was significantly ($p < 0.05$) increased from 27.5 μm to 44.4 μm, while a significant ($p < 0.05$) increase in size from 26.3 μm to 34.5 μm was observed as the pH was increased from 5.4 to 6.4. An increase in skim milk powder concentration level and a decrease in pH led to a significant ($p < 0.05$) increase in the encapsulation efficiency of probiotics. The viability of probiotics in a simulated stomach condition was increased when probiotics were encapsulated in milk protein-based delivery systems. An increase in the skim milk powder concentration and a decrease in pH resulted in an increase in the viability of probiotics in simulated stomach conditions. It was concluded that the protein content by modulating skim milk powder concentration level and pH were the key manufacturing variables affecting the physicochemical properties of milk protein-based probiotic delivery systems.



**Keywords (3 to 6):** milk protein, probiotics, delivery system, rennet

# Introduction

Probiotics can be defined as “live microorganisms which, when administered in adequate numbers, confer a health benefit on the host” (FAO/WHO, 2002). Owing to the beneficial effects of probiotics, such as reduction in the incidence and duration of childhood diarrhea, improvement in symptoms of the irritable bowel syndrome, and regulation of intestinal immunity,

demands for probiotic-formulated food products are increasing across the world [2, 3]. Probiotics have been incorporated in various foods including dairy products (e.g., yogurt, cheese, and ice cream) and non-dairy products (e.g., chocolate, cereals, and juices) and global probiotic market is expected to reach US$46.55 billion by 2020 [4-6]. However, there remain problems on the viability of probiotics when probiotics have been used in foods [4]. To provide their beneficial effects on the host, it is necessary that probiotics survive through the upper gastrointestinal tracts and sufficient numbers of probiotics reach the intestine alive [7]. To reach a sufficient number of viable probiotics to the intestinal epithelium, probiotic foods should contain at least $10^6$ - $10^7$ cfu/g at the time of consumption [8-10]. However, the number of viable cell in the intestinal epithelium is often low because the number of probiotic can be easily decreased under adverse and harsh conditions during food processing (e.g., heat treatment), storage, and digestion (e.g., acidic pH of stomach) [11]. Therefore, it is important to protect probiotics and keep them alive until reaching the intestine.

Microencapsulation technologies, such as spray-drying, spray-congealing, extrusion, and coacervation, have been used for the protection and delivery of probiotics [4, 12, 13]. Spray drying is regarded as a low cost process since it is suitable for high-volume production and requires low energy input., Therefore, spray drying has been widely used to encapsulate probiotics in various matrixes and to produce microparticles delivering probiotics [4]. However, high heat temperature (e.g., > 130℃) during spray drying negatively affected the viability of encapsulated probiotics [11, 14, 15]. Moreover, non-dairy origin delivery materials, such as alginate, gellan-gum, and xanthan, cannot be applied to dairy foods in some countries [11, 16]. Therefore, it is necessary to produce an effective delivery system for probiotics using dairy origin delivery materials, such as milk proteins with mild heat treatment.

Rennet is an enzymatic mixture with a protease activity. Chymosin, the major component of rennet, can hydrolyze (peptide bond between phenylalanine (residue 105) and methionine (residue106) in κ-casein [17]. The cleavage of κ-casein on the surface of casein micelles results in a decrease in the net negative charge and an increase in their hydrophobicity, which leads to aggregation of casein micelles and form a gel [11]. Heidebach et al. [11] produced dairy-based probiotic delivery system using the emulsification and rennet-induced gelation of skim milk. This method is relatively simple and suitable for both lab-scale and high volume manufacturing since specialist equipments are not necessary to use. However, in the study of Heidebach et al. (2009), very high concentration level (35%, w/w) of skim milk powder was used for the

encapsulation of probiotics, which can increase the production cost and lead to form larger particles (~69 μm). Although an increase in the size of delivery system could increase the encapsulation efficiency and viability of probiotics in previous studies [18, 19], larger delivery system may negatively affect the textural and sensorial properties of foods [4, 18]. Compared with spray drying method performed at high temperature above > 100℃, sub-ambient temperature treatment between 5 and 25℃ was used in rennet-induced gelation method, which could make it advantageous to minimize heat-induced damage for probiotics [20]. Moreover, milk proteins have lower viscosity than highly viscous non-dairy delivery materials including alginate, gellan-gum, and xanthan. Because of high viscosity of those non-dairy delivery materials even at low concentration, the developing non-dairy based gel networks can have the low density, which cannot offer efficient protection for encapsulated materials [11]. Therefore, rennet-induced gelation of milk proteins can be used as an ideal method for the encapsulation of probiotics.

To produce cost-effective probiotic delivery systems, the concentration level of skim milk powder should be reduced (e.g., below 10%). Moreover, to understand how the manufacturing processes can modulate the physicochemical characteristics of probiotic delivery systems including size and zeta potential, it is necessary to study the relationships between the manufacturing variables and physicochemical properties of probiotic delivery systems. In this study, we hypothesized that the manufacturing variables, such as milk protein concentration level and pH, that affect the rennet-induced gelation, may play important roles on the physicochemical properties of probiotic delivery systems, the encapsulation efficiency of probiotics, and viability of probiotics during gastrointestinal digestion.

The objectives of this study were to produce probiotic delivery system using rennet-induced gelation of milk proteins and to study how manufacturing variables, such as skim milk powder concentration level and pH, affect the physicochemical properties of probiotic delivery systems and the viability of encapsulated probiotics during *in vitro* gastrointestinal digestion.

# Materials and Methods

### Chemicals and Reagents

Skim milk powder with protein content of 35% (w/w) and rennet (Natural standard plus 290) were purchased from Maeil Dairies Co. Ltd. (Korea) and Hansen pty Ltd. (Newzealand), respectively. $CaCl_2$, Tween 80, Span 80, NaCl, KCl, $NaHCO_3$, pepsin from porcine gastric

mucosa, bile extract porcine, and Acridine orange were purchased from Sigma-Aldrich (St. Louis, USA).

**Microbial culture**

Probiotic strain, *L. rhamnosus* GG, was cultured in de Man, Rogosa, and Sharpe (MRS) media (Difco Laboratories, USA) at 37℃ for 18 h. After two subcultures in MRS media, cell suspension was centrifuged at 1,500 g, 4℃ for 5 min. The pellet was washed twice with sterile 0.9% (w/v) sodium chloride solution and used for further encapsulation process.

**Manufacture of probiotic delivery systems**

Probiotic delivery systems were produced using the modified rennet-induced gelation of milk proteins described in Heidebach *et al.* [11]. Skim milk solutions (3, 5, and 10% w/w) reconstituted in sterile water were adjusted to pH 5.4 and 6.2 using 1 M HCl and cooled to 5℃. The cell pellet was mixed with 15 mL of skim milk solutions with various concentration levels (3, 5, and 10%, w/w) and pH (5.4 and 6.2) to obtain probiotic/skim milk mixtures with at least 9.0 log CFU/mL of *L. rhamnosus* GG. Next, 51.7 μL of rennet was added to 15 mL of probiotic/skim milk mixtures and kept at 5℃ for 60 min. Seventy-five microliters of 1 M $CaCl_2$ were added to 15 mL of probiotic/skim milk/rennet mixtures. The final concentration of $CaCl_2$ in the mixture was 5 mM. To form oil-in-water (O/W) emulsions, 15 mL of those mixtures were added to 160 g of corn oil containing 5% (w/w) span 80 and then homogenized at 8,000 rpm for 5 min using a probe-type homogenizer (Daihan scientific Co., Ltd., Korea). To prevent rennet-induced gelation of milk proteins, temperature was kept at 5℃ during homogenization. After the formation of O/W emulsions, the temperature of emulsions was controlled to 25℃ and kept for 10 min to induce the rennet-induced gelation of milk proteins. To obtain probiotic delivery systems, O/W emulsions were centrifuged at 15,000 g, 4℃ for 1 min and then oils at top layer were removed. After washing 3 times with distilled water to remove residual oils, probiotic delivery systems were collected and freeze dried.

**Morphological properties of probiotic delivery systems**

Formation and morphological properties of probiotic delivery systems were determined using a confocal laser scanning microscope (CLSM, Olympus FV-1000, Japan). To monitor probiotic delivery systems, acridine orange, a fluorescent dye, was used to stain milk proteins. Prior to the

production of delivery systems, 90 μL of 0.2% (w/w) acridine orange was added to 15 mL of 3, 5, and 10% (w/v) skim milk solutions and adjusted to pH 5.4 and 6.2 with 1 M HCl followed by cooling to 5℃. Probiotic delivery systems were manufactured according to methods described above. After freeze drying, 0.15 g of probiotic delivery systems were placed on a concave confocal microscope slide. The excitation and emission wavelengths were set at 488 and 526 nm, respectively.

**Particle size and size distribution of probiotic delivery systems**

Particle size analyzer (1090LD shape, CILAS Co., Ltd., France) was used to measure the particle size (volume-mean diameter, D43) and size distribution (span value) of probiotic delivery systems. Prior to measure the size, 0.15 g of freeze-dried probiotic delivery systems were dispersed in 45 mL of 10% (w/w) tween 80 solution followed by sonication at 50 W for 3 min. Span value was calculated by following equation [21]

$$\text{Span value} = \frac{D\,(0.9) - D\,(0.1)}{D\,(0.5)}$$

where $D$ (0.9), $D$ (0.1), and $D$ (0.5) are particle diameter at cumulative size of 90%, 10%, and 50%, respectively. A low span value indicates uniformity in size distribution.

**Encapsulation efficiency of probiotics**

The encapsulation efficiency of probiotics in milk-protein based delivery systems was evaluated by counting viable cells in delivery systems using a standard plate method on MRS agar. Freeze-dried probiotic delivery systems were dispersed in 10% tween 80 solution and the number of viable cells was determined. To determine the number of viable cells in milk protein-based delivery systems, reconstituted delivery systems were enzymatically hydrolyzed using the modified method of Heidebach *et al*. [11]. Reconstituted delivery systems were diluted 10 times with Protease N "Amano" (0.0024 g/10 mL) and kept at 40℃ for 30 min with constant agitation at 150 rpm using a shaking water bath. After reaction with Protease N "Amano", viable cells were enumerated using a standard plate method on MRS agar. Encapsulation efficiency was calculated by following equation [22].

$$\text{Encapsulation efficiency} = N / N_0 \times 100$$

where $N_0$ is the initial number of *L. rhamnosus* GG added in the preparation process and N is the total number of *L. rhamnosus* GG encapsulated in probiotic delivery systems.

### Viability of encapsulated probiotics under simulated gastrointestinal conditions

The viability of encapsulated *L. rhamnosus* GG under simulated gastric and intestinal conditions was evaluated by the modified method of Chávarri *et al*. [23] and García-Sartal *et al*. [24]. To prepare simulated gastric juice (SGJ), pepsin (0.3%, w/v) was dissolved in 0.9% (w/v) NaCl solution and adjusted to pH 2.0. Simulated intestinal juice (SIJ) was prepared with 0.65% (w/v) NaCl, 0.0835% (w/v) KCl, 0.022% (w/v) $NaHCO_3$, and 0.3% (w/v) bile extraction porcine and then was adjusted to pH 7.5. SGJ and SIJ was filtered with a 0.45 μm syringe filter. Free and encapsulated probiotics (*L. rhamnosus* GG) were 10-fold diluted with SGJ and SIJ and then incubated at 37℃ for 120 min with constant agitation at 150 rpm using a shaking water bath (Daihan scientific Co., Ltd., Korea). After incubation, SGJ was diluted 10 times with 0.05 M sodium phosphate buffer (pH 7.0) for inactivation of pepsin. To determine the number of viable cells in milk protein-based delivery systems, delivery systems were enzymatically hydrolyzed using the modified method of Heidebach *et al*. [11] described earlier.

### Statistical analysis

All data were expressed as a mean of three replicates. The impacts of manufacturing variables, such as skim milk concentration level and pH, on the particle size and size distribution of probiotic delivery systems were determined by one-way analysis of variance (ANOVA) with Fisher's significant differences (LSD) test with statistical significance of $p < 0.05$. Repeated-measures ANOVA was used to determine the effects of manufacturing variables, incubation time, and their interactions on the survival of *L. rhamnosus* GG in simulated gastrointestinal conditions. The statistical analysis system (Version 9.1, SAS Institute Inc., USA) was used to perform ANOVA.



## Results and Discussion

### Morphological properties of probiotic delivery systems

Modified rennet-induced gelation method [11] was used to manufacture milk protein-based probiotic delivery systems. The formation and morphological properties of probiotic delivery systems were determined using CLSM (Figs. 1 and 2). Round shaped particles with a size ranging from 100 to 200 μm were observed indicating the successful development of probiotic delivery systems. An increase in the size of probiotic delivery systems was observed with an

199 increase in the skim milk powder concentration level from 3 to 10% (w/w) (Fig. 1) and in pH
200 from 5.4 to 6.2 (Fig. 2).
201
202 **Particle size and size distribution of probiotic delivery systems**
203 Impacts of skim milk powder concentration level and pH on the size of probiotic delivery
204 systems were assessed by using particle size analyzer (Fig. 3). As skim milk powder
205 concentration level was increased from 3 to 10% (w/w), the size of probiotic delivery system was
206 significantly ($p < 0.05$) increased from 27.5 to 44.4 μm (Fig. 3A). The cleavage of negatively
207 charged κ-casein molecule existed on the surface of casein micelles by rennet result in a
208 reduction in electrostatic repulsions between casein micelles. It can lead to an increase in the
209 aggregation of casein micelles resulting in the formation of gels [11]. Since more casein micelles
210 as building blocks may participate in the production of probiotic delivery system at higher skim
211 milk powder concentration level, their intermolecular associations could be increased with an
212 increase in skim milk powder concentration level, which may lead to the formation of thicker
213 and bigger protein network. This may result in an increase in the size of milk protein-based
214 probiotic delivery systems.
215 A decrease in pH from 6.2 to 5.4 resulted in a significant ($p < 0.05$) decrease in the size of
216 probiotic delivery systems (Fig. 3B). Because the isoelectric pH of casein micelles is ~4.6, a
217 decrease in pH from 6.2 to 5.4 lead to a decrease in the net negative charges of casein micelle
218 and therefore hydrophobic interactions between casein micelles could be increased [11, 17]. An
219 increase in the hydrophobic interactions between casein micelles may lead to an increase in the
220 intramolecular associations of protein gel network, which may result in the shrinkage of gel
221 network and formation of more compact and small particles at pH 5.4 [11, 25].
222 The size distribution of probiotic delivery systems was shown in Fig. 4. Span value is a
223 statistical parameter that can be useful to evaluate the distribution of particle size. Smaller the
224 span value indicates narrower size distribution (homogeneous) [21, 26]. In all conditions, span
225 values of probiotic delivery systems were ranged from 1.5 to 3.0 indicating that probiotic
226 delivery systems had narrow size distribution and formation of homogeneous particles [27].
227
228 **Encapsulation efficiency of probiotics**
229 Fig. 5 shows the effects of skim milk powder concentration level and pH on the encapsulation
230 efficiency of probiotics. An increase in the skim milk powder concentration level from 3 to 10%

(w/w) resulted in a significant ($p$<0.05) increase in the encapsulation efficiency of probiotics from 76.0 to 88.5% (Fig. 5A). An increase in skim milk powder concentration level may lead to the formation of bigger and thicker milk protein-based delivery systems (Fig. 3A). Therefore, it could provide more effective barriers to encapsulated probiotics resulting in an increase in the encapsulation efficiency of probiotics. Similar results were reported by Sheu *et al*. [18] and Lee *et al.* [28]. They reported that more probiotics were encapsulated in bigger delivery systems.

Although the particle size of probiotic delivery systems was decreased as pH was decreased from 6.2 to 5.4, encapsulation efficiency of probiotics was significantly ($p$<0.05) increased from 88.3 to 97.8% with a decrease in pH (Fig. 5B). Because same protein concentration level (5%, w/w) was used to manufacture those probiotic delivery systems at different pH, an increase in encapsulation efficiency may be due to increased intramolecular associations between protein molecules in rennet-induced milk protein gel networks. It is known that chymosin has the optimum pH for the hydrolysis of κ-casein, which is 5.1-5.3 [29, 30]. Imafidon and Farkye [31] also reported that the κ-casein cleavage by chymosin was greatest at pH 5.3-5.6. These results indicate that a decrease in pH from 6.2 to 5.4 could lead to an increase in the activity of chymosin on the hydrolysis of κ-casein. Since an increase in the hydrolysis of κ-casein could contribute to a decrease in electrostatic repulsions and an increase in hydrophobic attractions between casein micelles, more compact and denser protein networks could be formed at lower pH resulting in an increase in the encapsulation efficiency of probiotics [11, 32, 33].

**Viability of probiotics in simulated gastrointestinal conditions**

Impacts of encapsulation in milk protein-based delivery systems on the viability of probiotic stain, *L. rhamnosus* GG, during incubation under gastrointestinal conditions were presented in Table 1. Repeated measures ANOVA exhibited that encapsulation in milk protein-based delivery system (treat, $p < 0.0001$), incubation time (time, $p = 0.0005$), and their interaction ($p < 0.0001$) in simulated gastric juice (SGJ) had a significant effect on the viability of *L. rhamnosus* GG (Table 1). The number of viable cells was decreased during incubation in SGJ. A decrease in the viability of probiotics can be attributed to the high acidity of gastric juice under gastric conditions. When *L. rhamnosus* GG was encapsulated in milk protein-based delivery system, overall mean viable cell number was significantly ($p = 0.0005$) increased from 7.35 to 7.88 CFU/mL. It implies that encapsulation in milk protein-based delivery system is a useful method to enhance the viability of *L. rhamnosus* GG in gastric condition. When free and encapsulated *L.*

*rhamnosus* GG were exposed to simulated intestinal juice (SIJ), the repeated measures ANOVA results presented that encapsulation (treat, $p = 0.0934$), incubation time (time, $p = 0.8153$), and their interaction ($p = 0.7306$) were not significant indicating that bile salts and intestinal pH did not affect the viability of *L. rhamnosus* GG was not affected under intestinal juice condition, such as bile salts and intestinal pH.

Table 2 shows the impacts of manufacturing variables, such as skim milk powder concentration level and pH on the viability of encapsulated probiotics, *L. rhamnosus* GG, in simulated gastrointestinal conditions. Repeated measures ANOVA revealed significant effects on the skim milk powder concentration level (treat, $p = 0.0001$), incubation time ($p < 0.0001$), and their interaction ($p < 0.0001$) on the viability of *L. rhamnosus* GG in SGJ while no significant effects were observed in SIJ (Table 2). As skim milk powder concentration level was increased from 3 to 10% (w/w), the overall viability of *L. rhamnosus* GG in SGJ was increased from 7.34 to 7.69 CFU/mL indicating that skim milk powder concentration level can be a key factor to enhance the viability of probiotics in gastric condition. An increase in protein content by increasing skim milk powder concentration level may enhance the intermolecular associations between casein micelles resulting in the production of thicker protein networks. Those thicker protein gel networks could reduce the diffusion of gastric juice with very high acidity into the probiotic delivery systems [34]. Therefore, delivery systems prepared with higher milk protein concentration level can provide better protections for probiotics in harsh gastric conditions compared with delivery systems treated with lower milk protein concentration level. Similar results were reported by Shi et al. [34] who presented that probiotic delivery systems produced with higher milk concentration could provide better protections for *L.bulgaricus* in SGJ

In repeated measures ANOVA, a significant effect of pH (treat, $p < 0.0001$), incubation time ($p < 0.0001$), and their interaction ($p = 0.0003$) on the viability of of *L. rhamnosus* GG in SGJ was observed while there were no significant effects observed in SIJ (Table 2). Overall viability of *L. rhamnosus* GG in SGJ was increased from 7.88 to 8.33 CFU/mL as pH was decreased from 6.2 to 5.4. As we described earlier, a decrease in pH 6.2 to 5.4 may enhance hydrophobic attractions between casein micelles forming more compact and denser protein gel networks. The production of denser protein gel networks could reduce acid diffusions into milk protein-based delivery systems in SGJ, which may contribute to higher viability of *L. rhamnosus* encapsulated in milk protein-based delivery system prepared at pH 5.4.

In conclusion, probiotic strain, *L. rhamnosus* GG, was successfully encapsulated in milk protein-based delivery systems using a modified rennet-induced gelation method. Skim milk powder concentration level and pH were major manufacturing variables affecting the physicochemical properties of milk protein-based probiotic delivery systems, such as a particle size, size distribution, encapsulation efficiency, and viability of probiotics in simulated gastrointestinal tract. It can be attributed to intra- and intermolecular associations between milk protein molecules during rennet-induced gelation. Milk protein-based delivery systems can be used for enhancing the viability of probiotics and these food-grade probiotic delivery systems can be useful to apply probiotics to various foods.

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**If a DOI (digital object identifier) is available for an article, always include it.**


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34. Shi LE, Li ZH, Li DT, Xu M, Chen HY, Zhang ZL, Tang ZX. Encapsulation of probiotic *Lactobacillus bulgaricus* in alginate–milk microspheres and evaluation of the survival in simulated gastrointestinal conditions. J Food Eng. 2013;117:99-104. https://doi.org/10.1016/j.jfoodeng.2013.02.012**Table 1.** Effects of probiotic encapsulation in milk protein-based delivery systems on the viability of probiotics during incubation in simulated gastric juice and simulated intestinal juice [1].

| Time (min) | Free probiotic | Encapsulated probiotic [2] |
|---|---|---|

| | | | |
|---|---|---|---|
| SGJ [3] | 0 | 8.97 | 8.91 |
| | 30 | 7.92 | 8.01 |
| | 60 | 6.70 | 7.63 |
| | 120 | 5.82 | 6.98 |
| | Overall (0-120) [5] | 7.35 | 7.88 |
| | Pooled SD [6] | 0.11 | |
| | P-value | | |
| | Treat [7] | 0.0005 | |
| | Time [8] | < 0.0001 | |
| | Treat × Time [9] | < 0.0001 | |
| SIJ [4] | 0 | 8.97 | 8.91 |
| | 30 | 8.87 | 8.94 |
| | 60 | 8.84 | 8.92 |
| | 120 | 9.02 | 8.91 |
| | Overall (0-120) | 8.93 | 8.92 |
| | Pooled SD | 0.15 | |
| | P-value | | |
| | Treat | 0.9034 | |
| | Time | 0.8153 | |
| | Treat × Time | 0.7306 | |

[1] Data are mean values of triplicates and are expressed as $\log_{10}$ CFU/mL.

[2] Probiotics (*L. rhamnosus* GG) were encapsulated in milk protein-based delivery system prepared with 5% (w/w) skim milk powder at pH 6.2.

[2] SGJ: simulated gastric juice.

[3] SIJ: simulated intestinal juice.

[4] Overall (0-16): mean values of overall incubation period.

[5] Pooled SD: pooled standard deviation

[6] Treat: probiotic encapsulation in milk protein-based delivery systems.

[7] Time: incubation time in minutes.

[8] Treat × Time: interaction between treat and time.

**Table 2.** Impacts of skim milk powder concentration level and pH on the viability of encapsulated probiotics during incubation in simulated gastric juice and simulated intestinal juice [1].

| Time (min) | skim milk powder concentration level (%, w/w) [2] | | | pH[3] | |
|---|---|---|---|---|---|
| | 3 | 5 | 10 | 5.4 | 6.2 |

| | | | | | | |
|---|---|---|---|---|---|---|
| SGJ [4] | 0 | 8.94 | 8.91 | 9.00 | 8.96 | 8.91 |
| | 30 | 7.81 | 8.01 | 8.01 | 8.72 | 8.01 |
| | 60 | 6.57 | 7.63 | 6.84 | 7.96 | 7.63 |
| | 120 | 6.04 | 6.98 | 6.92 | 7.70 | 6.98 |
| | Overall (0-120) [6] | 7.34 | 7.88 | 7.69 | 8.33 | 7.88 |
| | Pooled SD [7] | 0.11 | | | 0.09 | |
| | P-value | | | | | |
| | Treat [8] | 0.0001 | | | <0.0001 | |
| | Time [9] | < 0.0001 | | | < 0.0001 | |
| | Treat × Time [10] | < 0.0001 | | | 0.0003 | |
| SIJ [5] | 0 | 8.94 | 8.91 | 9.00 | 8.96 | 8.91 |
| | 30 | 8.87 | 8.94 | 8.94 | 8.72 | 8.94 |
| | 60 | 9.07 | 8.92 | 9.01 | 7.96 | 8.92 |
| | 120 | 8.92 | 8.91 | 9.00 | 7.70 | 8.91 |
| | Overall (0-120) | 8.95 | 8.92 | 8.99 | 8.33 | 8.92 |
| | Pooled SD | 0.11 | | | 0.10 | |
| | P-value | | | | | |
| | Treat | 0.2581 | | | 0.3575 | |
| | Time | 0.6490 | | | 0.8375 | |
| | Treat × Time | 0.8613 | | | 0.8949 | |

[1] Data are mean values of triplicates and are expressed as $\log_{10}$ CFU/mL.
[2] Probiotics (*L. rhamnosus* GG) were encapsulated in milk protein-based delivery system prepared with various skim milk powder concentration levels (3, 5, and 10%, w/w) at pH 6.2.
[3] Probiotics (*L. rhamnosus* GG) were encapsulated in milk protein-based delivery system prepared with skim milk powder concentration level of 5% (w/w) at various pH (5.4 and 6.2).
[4] SGJ: simulated gastric juice.
[5] SIJ: simulated intestinal juice.
[6] Overall (0-16): mean values of overall incubation period.
[7] Pooled SD: pooled standard deviation
[8] Treat: probiotic encapsulation in milk protein-based delivery systems.
[9] Time: incubation time in minutes.
[10] Treat × Time: interaction between treat and time.

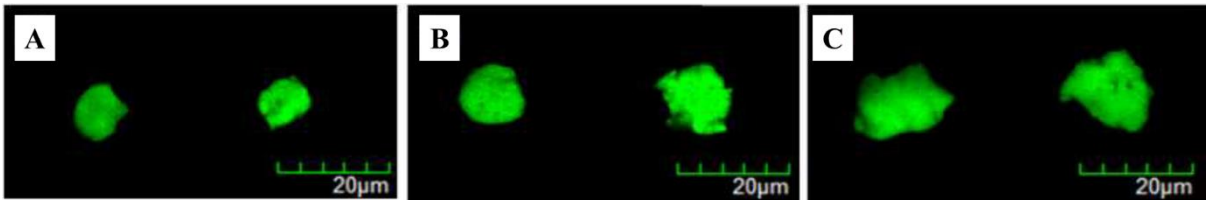


**Fig. 1.** Morphological properties of probiotic delivery systems prepared with various skim milk powder concentration level. Probiotic delivery systems were manufactured with 3 (A), 5 (B), and 10% (w/w) (C) of skim milk powder concentration levels at pH 6.2. Scale bar = 20 μm.

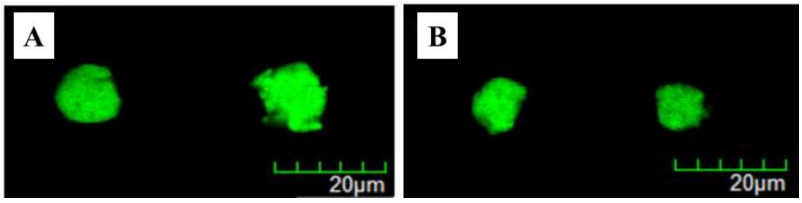


**Fig. 2.** Morphological properties of probiotic delivery systems prepared at various pH. Probiotic delivery systems were manufactured 5% (w/w) of skim milk powder concentration level at pH 6.2 (A) and 5.4 (B). Scale bar = 20 μm.

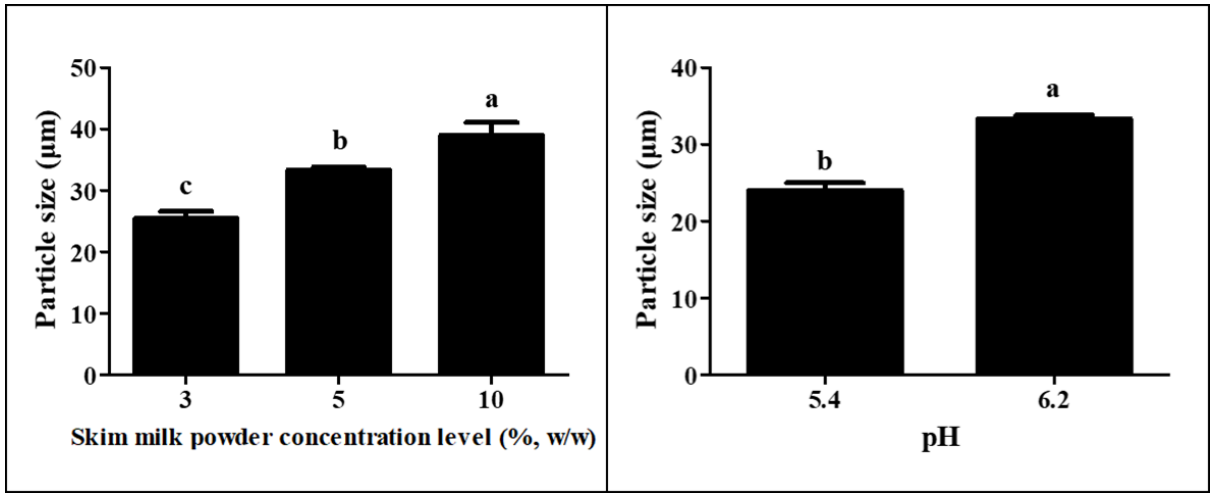


**Fig. 3.** Impacts of skim milk powder concentration level and pH on the size of probiotic delivery systems: (A) Probiotic delivery systems were manufactured with 3, 5, and 10% (w/w) skim milk powder concentration level at pH 6.2. (B) Probiotic delivery systems were manufactured with 5% (w/w) skim milk powder concentration level at pH 5.4 and 6.2. Different letters on a column indicate significant ($p<0.05$) differences.

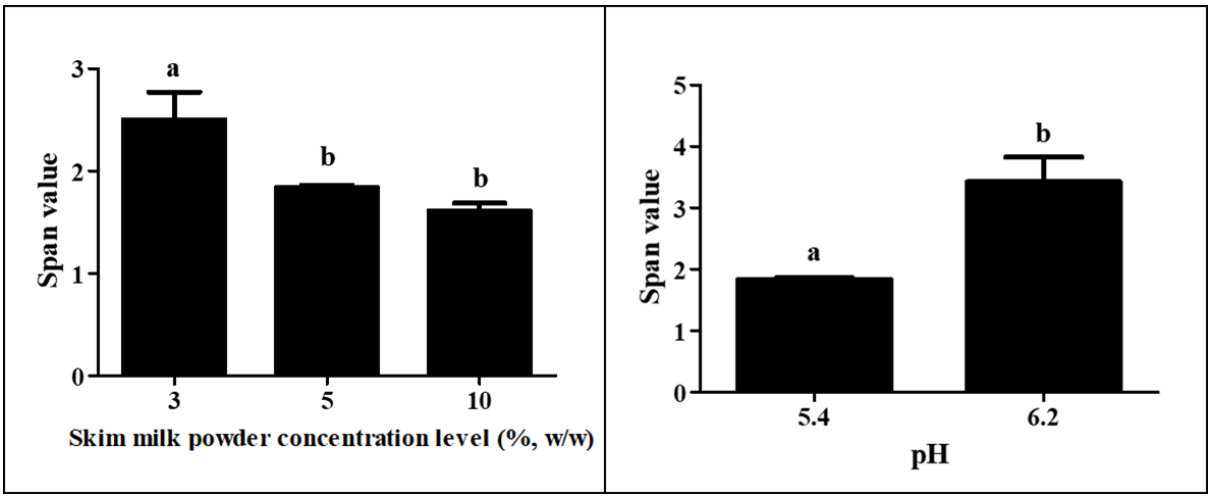


**Fig. 4.** Effects of skim milk powder concentration level and pH on the span value of probiotic delivery systems: (A) Probiotic delivery systems were manufactured with 3, 5, and 10% (w/w) skim milk powder concentration level at pH 6.2. (B) Probiotic delivery systems were manufactured with 5% (w/w) skim milk powder concentration level at pH 5.4 and 6.2. Different letters on a column indicate significant ($p<0.05$) differences.

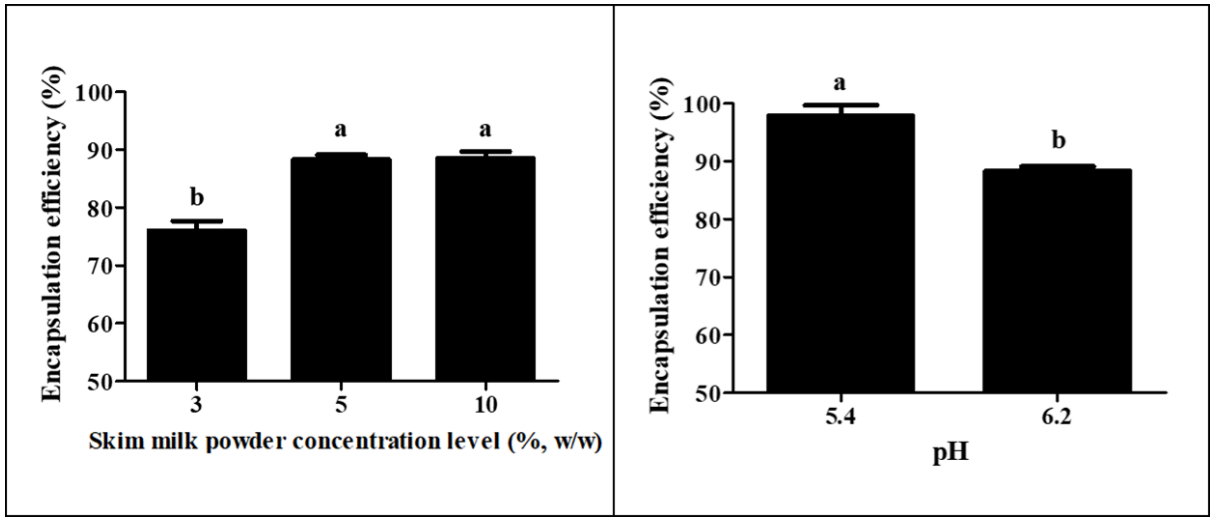


**Fig. 5.** Impacts of skim milk powder concentration level and pH on the encapsulation efficiency of probiotics: (A) Probiotic delivery systems were manufactured with 3, 5, and 10% (w/w) skim milk powder concentration level at pH 6.2. (B) Probiotic delivery systems were manufactured with 5% (w/w) skim milk powder concentration level at pH 5.4 and 6.2. Different letters on a column indicate significant ($p<0.05$) differences.