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Effect of alginate and chitosan on viability and release behavior of *Bifidobacterium pseudocatenulatum* G4 in simulated gastrointestinal fluid

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ABSTRACT

The main aim of this study was to investigate the effect of different coating materials (i.e. Na-alginate and chitosan) on the viability and release behavior of *Bifidobacterium pseudocatenulatum* G4 in the simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). This study reports the viability of encapsulated *B. pseudocatenulatum* G4 coated using different alginate (2–4 g/100 mL) and chitosan (0.2–0.8 g/100 mL) concentrations. The results indicated that the highest concentration of alginate (4.4142 g/100 mL) along with 0.5578 g/100 mL chitosan resulted in the highest viability of *B. pseudocatenulatum* G4. The release behavior of the encapsulated probiotics in SGF (pH 1.5) in 2 h followed by 4 h in SIF (pH 7.4) was also assessed. The resistance rate of alginate–chitosan capsule in SGF was higher than SIF. The alginate–chitosan encapsulated cells had also more resistance than alginate capsules. The current study revealed that alginate encapsulated *B. Pseudocatenulatum* G4 exhibited longer survival than its free cells (control).

1. Introduction

Probiotics' bacteria have beneficial effects for humans. They can promote feasible biological roles in host (human-body). They can also provide much further protection to the human health via neutralizing harmful products from foods in the gastrointestinal tract (Ray, 2004). The probiotics must remain viable to confer these benefits, hence it hinges on their survival through the critical stages of processing and gastrointestinal transit (Cheow, Kiew, & Hadinoto, 2014). Bifidobacterium is one of well-known probiotics because of its beneficial effect on the human intestinal tract (Hughes & Hoover, 1991). It controls the acidity of the large intestinal tract. It is also capable of hydrolyzing lactulose or other indigestible complex carbohydrate into acetic and lactic acids, thus maintaining the intestinal microbial balance by barring the growth of potential pathogens (Rasic & Kurman, 1983). The consumption of probiotics is highly recommended in high concentration (10^6–10^7 viable cells/g) (Shah & Ravula, 2000).

The oral administration of probiotics induces very useful functions in clinical trials. Probiotics have been used for relief of specific diseases of the gastrointestinal tract. However, probiotic cells should be alive to exert their health benefits (Cook, Tzortzis, Khutoryanskiy, & Charalampopoulos, 2013). The number of viable probiotic cells reduces because of low pH of the stomach. Consequently, this will reduce the efficacy and function of the probiotic in the gastrointestinal tract (Cook et al., 2013). Therefore, the enhancement of the viability of these useful bacteria in food products is one of the most important issues in food industry. Encapsulation technique is mainly used to improve the viability of probiotics through packaging them into small, sealed capsules. The encapsulation can control the release of probiotics under specific conditions (Anal, Stevens, & Lopez, 2006). This extends the shelf life of products through increasing the viability of probiotics. Lankaputhra and Shah (1995) reported that many strains of Bifidobacterium species did not have the ability to survive in human gastrointestinal tract (GIT). This is because of the harmful impact of acidity and bile concentration in GIT. Nevertheless, the application of the suitable entrapment technique for Bifidobacterium using appropriate coating materials is highly encouraged.

The most important factors affecting the viability of probiotics are acidity, concentrations of lactic and acetic acids, storage temperature, dissolved oxygen content, hydrogen peroxide, type and

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content of encapsulating agents (Krasaekoopt, Bhandari, & Deeth, 2003; Pania & Nath, 2014; Picot & Lacroix, 2004). Alginate is a natural polysaccharide from brown algae. It comprises a linear chain of 1–4 linked β-D-mannuronic acid (M) and R-L-guluronic acid (G) residues (Kuo and Ma (2001). Alginate exists as a polyanion in solution because of the presence of carboxylic groups on both monomers (Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2011). Calcium alginates are the most commonly coating material for the entrapment of probiotics. Alginate beads are found capable to easily form gel matrices around bacterial cells. They are established to increase the survival of probiotics up to 95% in several other researches. In the present of chelators and non-gelling cations, the gelation efficiency of alginate is not satisfactory to increase the viability of probiotic cells. Therefore, capsules are coated with polycationic polymers to enhance the viability and stability of alginate.

Chitosan is the cationic (1–4)-2-amino-2-deoxy-β-d-glucan produced from chitin as the second most abundant polysaccharide in nature (Muzzarelli, 2012). Chitosan polymers are often used as coats/shells rather than capsules. They can be further polymerized by means of cross-link formation in the presence of anions and polyanions (Klein, Stock, & Vorlop, 1983; Muzzarelli, Stanic, Gobbi, Tosi, & Muzzarelli, 2004). Glucosamine, chitooligomers and chitosans are directed orally for dietary and drug delivery purposes to healthy subjects and patients (Bottegoni, Muzzarelli, Busilacchi, Giovannini, & Gigante, 2014; Muzzarelli & Muzzarelli, 2006). In general, the diluted chitosan (e.g. 0.4%) is used for shell-making on alginate capsules, resulting in chitosan-coated alginate particles (Zhou, Martins, Groboilloot, Champagne, & Neufeld, 1998).

The main objective of the present study was to investigate the effect of different coating materials (i.e. Na-alginate and chitosan) on the viability and release behavior of encapsulated *Bifidobacterium pseudocatenulatum* G4 in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). It should be noted that alginate and chitosan have been used as coating materials for microencapsulation and protection of several probiotics (such as *Bifidobacterium bifidum*, *B. breve and Lactobacillus gasseri*) (Chávarri et al., 2010; Cook et al., 2011, 2013; Zhang, Li, Park, & Zhao, 2013). As mentioned by Cook et al. (2011), both polymers (i.e. chitosan and alginate) are complex biopolymers with high charge density. Hence, they can extend the capsule's residence in the area of release. Consequently, they control the release of the probiotic in the human intestinal tract. The chitosan–alginate capsules with the highest cell protection efficiency were targeted for the release behavior of *Bifidobacterium* G4 in the simulated GIT.

2. Materials and methods

2.1. Microorganisms

The stock culture of *B. pseudocatenulatum* G4 was achieved from stock culture collection of Food Biotechnology and Functional Food Laboratory, Faculty of Food Science and Technology, Universiti Putra Malaysia (UPM). It was activated by spreading on de Mann, Rogosa, Sharp (MRS) + L-cysteine agar plate. Then, it was incubated at 37 °C for 48 h under anaerobic condition. Subsequently, the strain was sub-cultured on other MRS + L-cys agar plate three times. Anaerocult® A (Merck, Darmstadt, Germany) in an anaerobic jar (BBL, Rockville) was used to produce anaerobic condition. A single colony of the target strain was chosen, and transferred from MRS + cys agar to MRS + cys broth. In the subsequent step, the centrifuge tube containing the inoculate cultures was incubated at 37 °C for 48 h. Anaerocult® A was used to maintain anaerobic condition for incubated cultures. The tubes were kept in the anaerobic jar. Finally, 10% (v/v) of the inoculate cultures was transferred to MRS + cys broth for 24 h for preparation approximately 10^{10} cfu/mL. Then, it was centrifuged at 11,068 × *g* for 15 min at 4 °C. The initial cell concentration was kept constant throughout this experiment (10^{10} cfu/mL) (Stephenie, Kabir, Shuhaimi, Rosfarizan, & Yazid, 2007).

2.2. Encapsulation of Bifidobacterium pseudocatenulatum G4

The encapsulation of *B. pseudocatenulatum* G4 was carried out according to the previous method (Sheu & Marshall, 1993). Probiotic-loaded capsules were prepared by using different concentrations of alginate (2–4 g/100 mL). Alginate solutions were heated at the autoclave (121 °C, 15 min). After autoclaving process, the solution was ready to cool down to 38–40 °C. Approximately, 1×10^{10} bacteria cells were mixed with alginate (1:4, v/v). Then, 20 mL of cell mixture was added to 100 mL of vegetable oil in 800 mL beaker. Tween 80 (2% w/w) was added to the oil as an emulsifier. The mixture was stirred at 200 rpm to make the uniform turbid emulsion. It was obtained within 10 min with no evidence of a free aqueous phase. Calcium chloride (100 mL 0.1 M) was then added carefully (20 mL/s) from the down side of the beaker until the water in oil (W/O) emulsion was separated. After calcium chloride was added, the soluble alginate became insoluble and consequently separated in the aqueous phase. Smaller particles of the water phase in water in oil (W/O) emulsion lead to the formation of beads with smaller diameters. Calcium-alginate beads were formed within 20 min. Finally, they were collected by centrifugation (350 × *g*, 10 min) and washed with sterile water. The capsules were stored in 0.1% (v/v) peptone water at 4 °C.

2.3. Coating of alginate beads with chitosan

Coating deposits an additional membrane-layer on the capsule surface. This leads to increase the mechanical strength and a more pronounced barrier function (Krasaekoopt, Bhandari, & Deeth, 2004). This is typically achieved by immersing the hydrogel capsules into a solution of coating polymer. Alginate beads were coated with chitosan according to the previous method (Krasaekoopt et al., 2004). Four different concentration of chitosan (0.2, 0.4, 0.6, and 0.8 g/100 mL) were used as a multiple coat. Low molecular weight chitosan (75–85% degree of acetylation) was used for encapsulation purpose (Zhou et al., 1997). An aqueous chitosan solution was prepared by dissolving 0.4 g chitosan in 90 mL distilled water acidified with 0.4 mL of glacial acetic acid (0.1 N) (Merck, Darmstadt, Germany). This was to achieve a final chitosan concentration of 0.4 (g/100 mL, w/v). Chitosan solution was autoclaved at 121 °C for 15 min. After dissolution, 1 mol L/L (1 N) NaOH was added to adjust pH (~5.7–6.0). The solution was filtered (Whatman no. 4) and the volume was adjusted to 100 mL. Alginate beads with cells were washed with distilled water and immersed (about 12–15 g) in 100 mL of chitosan solution. Then, it was gently stirred at 100 rpm for 40 min on an orbital shaker. Then alginate–chitosan was filtered and washed with sterile peptone water and stored at 4 °C.

2.4. Survival assessment evaluation and enumeration of encapsulated B. pseudocatenulatum G4 probiotic

Depolymerization technique was used to soften the bacteria capsules and facilitate the release of probiotic cells into the solutions. Approximately 1 g of capsules was exposed to depolymerization solution according to the previous method (Sheu & Marshall, 1993). The solution containing 180 mL of 0.2 M Na_2HPO_4 and 70 mL of 0.2 M solution of NaH_2PO_4 was adjusted to 500 mL with distilled water using volumetric flask. Then the flask was incubated at 37 °C for 10 min. The purpose of incubation was to soften the probiotic capsules for further release similarly to the human's body temperature. Final pH of phosphate buffer was approximately

6.8. After incubation with the depolymerization solution, a Polytron homogenizer (Brinkman Instruments, Rexdale, Canada) was employed for releasing the encapsulated *B. pseudocatenulatum* G4 via homogenization (Truelstrup Hansen et al., 2002). The encapsulated *B. pseudocatenulatum* G4 with high concentration of alginate and chitosan was released through homogenization. The released *B. pseudocatenulatum* G4 was serially diluted up to 10-fold in order to obtain the suitable concentration. Then, it was placed on MRS agar supplemented with 0.5 g/L L-cysteine hydro chloride. Subsequently, the plates were incubated in an anaerobic chamber for 48 h and the viability of encapsulated *B. pseudocatenulatum* G4 was expressed as log cfu/mL.

2.5. Preparation of simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)

Both SGF and SIF solutions were made according to the instruction reported earlier (USP, 2007; Nieddu et al., 2014) with minor modification. To prepare simulated gastric fluid (SGF), 2.0 g of sodium chloride and 3.2 g purified porcine derived pepsin were added to 7 mL of the concentrated hydrochloric acid and mixed to be dissolved in the acid. Then, the distilled water was added to the solution. After adjusting the volume up to 1000 mL, pH was approximately 1.5. The membrane filter (0.45 μm) was used to sterilize the mixture. Simulated intestinal fluid (SIF) was prepared by adding 6.8 g of KH_2PO_4 to 250 mL of distilled water and mixed well. Then, 10 g of pancreatin (Sigma–Aldrich, Inc., St. Louis, MI, USA) and 77 mL of 0.2 NaOH were mixed. The volume was adjusted to 1000 mL with distilled water. Finally, pH of the solution was adjusted to 6.8 with either 0.2 N NaOH or 0.2 NHCl and solution was sterilized with the membrane filter (0.45 μm).

2.6. Release of B. pseudocatenulatum G4 from alginate–chitosan capsules in SGF and SIF

The release behavior of *B. pseudocatenulatum* G4 from the capsules in GIF was studied according to the method described by Xaioyan and Xiguang (2009). Approximately, 1 g of the capsules was added to 50 mL SGF (pH 1.5). Then it was incubated at 37 °C for 30, 60, 90 and 120 min while shaking at 110 rpm. Subsequently, it was continuously transferred into SIF (pH 7.4) and sieved for 4 h. A spectrophotometer (Hitachi U 2800, Tokyo, Japan) was employed for release study. At particular time interval (every 30 min), 2.0 mL aliquots was collected and subjected to OD 600 assay. The beads stability was carried out in triplicates for each sample. In this experiment, the sterile peptone saline was used as a control. After releasing encapsulated cells in every 30 min of exposure to simulated gastric, the live bacteria were enumerated.

2.7. Statistical analysis

A central composite design (CCD) was employed to optimize the viability of *B. pseudocatenulatum* G4 after releasing from capsules as a response (Table 1). In this design, eight factorial points and six center points fall inside the range of independent variables. The presence of six star points outside the studied ranges can help the researchers to find out the possible unexpected changes of the response beyond the applied ranges (Mirhosseini & Tabatabaee Amid, 2012). The experimental design was developed to (i) find a relationship between each response and three independent variables and (ii) to determine the optimum level of the independent variables resulting in the desirable goals (Mirhosseini, Tan, Hamid, & Yusof, 2007). Regression equation and analysis of variance (ANOVA) performed to determine regression coefficients with statistical significance of model terms and to fit the mathematical models to the experimental data (Tabatabaee Amid & Mirhosseini, 2012). The following model was used to describe the variation of variability as function of the concentration of coating material (Mirhosseini et al., 2008a):

$$Y = \beta_0 + \sum \beta_1 x_1 + \sum \beta_2 x_2 + \sum \beta_{11} x_1^2 + \sum \beta_{22} x_2^2 + \sum \beta_{12} x_1 x_2$$

where Y is response calculated by the model; β_0 is a constant; and β_1, and β_2 are the main single coefficients of alginate and chitosan, β_{11}, and β_{11} are the quadratic coefficients of independent variable variables (Mirhosseini, Tan, Taherian, & Boo, 2009); while β_{12} is the interaction coefficient between alginate and chitosan, respectively. Only terms found statistically significant ($p < 0.05$) were included in the reduced model. In the present study, the percentage of two different encapsulating agents (i.e. chitosan and alginate) was considered as independent variables. The viability of the target bacteria was considered as the response variable. The confident level of 95% was used to run the significant test (Mirhosseini & Tabatabaee Amid, 2013). MINITAB software (Version 14, Minitab Inc., State College, PA, USA) was used to create the experimental design and further data analysis.

Table 1
Matrix of central composite design (CCD).

Run order	Blocks	Independent variables		Viability (log cfu/mL)
		Chitosan	Alginate	
1[a]	2	0.50	3.00	6.53
2[a]	2	0.50	3.00	6.59
3	2	0.92	3.00	6.26
4[a]	2	0.50	3.00	6.51
5	2	0.50	4.41	8.65
6[a]	2	0.50	3.00	6.53
7	2	0.08	3.00	5.80
8[a]	2	0.50	3.00	6.52
9	2	0.08	3.00	5.73
10[a]	2	0.50	3.00	6.32
11	2	0.50	1.59	4.94
12	2	0.50	1.59	4.95
13	2	0.92	3.00	6.24
14	2	0.50	4.41	8.61
15[a]	1	0.50	3.00	6.43
16	1	0.20	4.00	6.63
17[a]	1	0.50	3.00	6.49
18	1	0.20	2.00	4.91
19	1	0.80	2.00	5.15
20	1	0.20	4.00	6.69
21[a]	1	0.50	3.00	6.57
22[a]	1	0.50	3.00	6.55
23	1	0.80	4.00	6.99
24	1	0.20	2.00	5.00
25[a]	1	0.50	3.00	6.49
26	1	0.80	2.00	5.08
27[a]	1	0.50	3.00	6.63
28	1	0.80	4.00	7.01

[a] Center point.

3. Results and discussion

3.1. Effect of encapsulating/coating materials on viability of B. pseudocatenulatum G4

Alginate–chitosan is one of the most widely used microcapsules for microbial cultures. It is developed due to the appropriate compatibility, low cost and chitosan abundance in nature. Table 1 shows the experimental design involving both independent and response variables and all treatment runs. It should be noted that some variables were kept in the final reduced model despite their insignificance effect (Mirhosseini et al., 2008b). For example, main terms were also kept in the final reduced model if a quadratic or interaction term containing this variable was significant ($p < 0.05$)

Table 2
Regression coefficients and analysis of variance of the reduced regression models for viability of *B. pseudocatenulatum* G4 cells.

Term[a]	β (regression coefficient)
Constant	1.9507
x_1	1.0996
x_2	4.6163
x_1^2	–
x_2^2	−4.1271
x_1x_2	–
Regression	0.000*
Lack of fit	0.007*
R^2 (%)	0.95%

[a] x_1: alginate concentration (g/100 mL); x_2: chitosan concentration (g/100 mL).
* Significant at $p < 0.05$.

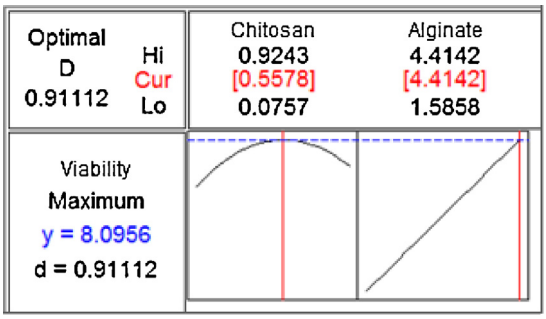

Fig. 2. Response optimizer graph showing the predicted optimum point.

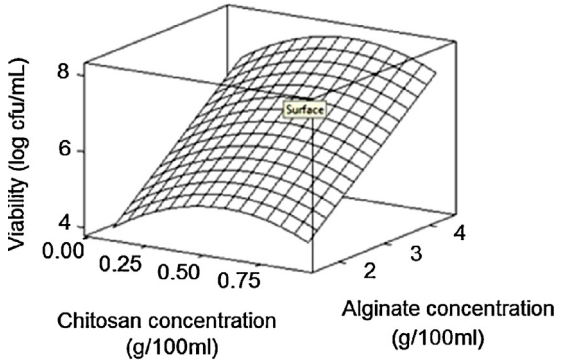

Fig. 1. Response surface plots showing the interaction effect of alginate and chitosan concentration (g/100 mL) on the viability of *Bifidobacterium pseudocatenulatum* G4.

(Mirhosseini & Tan, 2009). *F*-ratio and *p*-value of linear, quadratic and interaction effects of alginate and chitosan concentrations on the response variable are displayed in Table 2. The quadratic term of chitosan concentration followed by the main effects of alginate and chitosan concentrations had the most significant ($p \leq 0.05$) effects on the viability of encapsulated *B. pseudocatenulatum* G4, while the interaction effects of alginate and chitosan and the quadratic term of alginate had no significant ($p > 0.05$) effects on the viability of encapsulated cells (Table 3).

Fig. 1 illustrates the effects of alginate and chitosan concentration on the viability of encapsulated *B. pseudocatenulatum* G4 before exposing to SGF. The surface plot indicated that the increase of chitosan concentration up to 0.56 g/100 mL enhanced the viability of encapsulated *B. pseudocatenulatum* G4. However, the microencapsulation using high concentration of chitosan (>0.56 g/100 mL) had a negative effect on the viability of *B. pseudocatenulatum* G4. As shown in Fig. 1, the viability of encapsulated *B. pseudocatenulatum* G4 improved with increasing the concentration of alginate. Survival of encapsulated cells prolonged with increasing alginate concentration from 2 to 4 (g/100 mL). However, the increase of alginate concentration had no effect on releasing of entrapped cells in solution of colonic pH.

The release amount of alginate–chitosan complex beads into dissolution medium increased with decreasing the concentration of alginate in the microencapsulation process. This might be due to the reduction of cross-linking density of the beads. Formations of stronger binding linkage of chitosan–alginate complex membrane in the outer layer might be responsible for the reduction of release rate (Mi, Sung, & Shyu, 2002). Gåserød, Sannes, and Skjak-BraeK (1998) reported that alginate concentration in the gel affected the pore size of the capsules. They also found that high concentration of alginate resulted in more homogeneous capsules. As a result, homogeneous gel beads were expected to bind chitosan faster than the non-homogeneous gel. This could be due to a much lower surface alginate in non-homogeneous capsules (Gåserød et al., 1998). The result showed that the application of the highest concentration of alginate (4.41 g/100 mL) resulted in the highest viability of *Bifidobacterium G4* cells (Figs. 1 and 2). The present data confirmed the optimum point predicted by RSM. The results revealed that the viability of *B. pseudocatenulatum* G4 improved from 4.945 (±0.005) to 8.630 (±0.020) (log cfu/mL) with increasing alginate concentration from 1.59 to 4.41 (g/100 mL) (Table 1). This indicates that it is possible to have higher viability with increasing alginate concentration at the recommended optimum chitosan concentration (4.41 g/100 mL). This observation was also reported by previous researchers (Mandal, Puniya, & Singh, 2005).

In the present study, predicted and experimental (triplicate) values were compared using *t*-test to verify the validity of final reduced model (Table 4). The adequacy of the response surface equation was checked by a comparison between experimental and predicted values based on the reduced response regression models (Mirhosseini et al., 2007). Both experimental and predicted values of viability are shown in Table 4. No significant difference ($p > 0.05$) was observed between experimental and predicted values, thus verifying the adequate fitness of the final reduced model fitted for prediction of the viability of *B. pseudocatenulatum G4* as a function of chitosan and alginate concentration.

Table 3
Significant probability (*p*-values and *F*-ratio) of the independent variables effects in the final reduced models.

Variable		Main effects[a]		Quadratic effects[b]		Interaction effects[c]
		x_1	x_2	x_1^2	x_2^2	x_1x_2
Viability	*p*-value	0.000*	0.000*	–	0.000*	–
	F-ratio	378.03	46.24	–	40.06	–

[a] x_1 and x_2: the main effect of alginate and chitosan concentration, respectively.
[b] x_1^2 and x_2^2: the quadratic effect of alginate and chitosan concentration (g/100 mL), respectively.
[c] x_1x_2: the interaction effect alginate and chitosan concentration.
* Significant at $p \leq 0.05$.

Table 4
Comparison between experimental and predicted values based on the final reduced models.

Treatment	Viability (log cfu/mL)		
	Y_0 (experimental value)	Y_i (predicted value)	$Y_0 - Y_i$ (residue)
1[a]	6.53	6.653	−0.128
2[a]	6.59	6.653	−0.062
3	6.26	6.117	0.138
4[a]	6.51	6.653	−0.148
5	8.64	8.208	0.436
6[a]	6.53	6.653	−0.121
7	5.80	5.702	0.097
8[a]	6.52	6.653	−0.134
9	5.73	5.702	0.030
10[a]	6.32	6.653	−0.330
11	4.94	5.098	−0.158
12	4.95	5.098	−0.143
13	6.24	6.117	0.118
14	8.61	8.208	0.405
15[a]	6.43	6.399	0.032
16	6.63	6.981	−0.347
17[a]	6.49	6.399	0.092
18	4.91	4.781	0.127
19	5.15	5.075	0.074
20	6.69	6.981	−0.291
21[a]	6.57	6.399	0.169
22[a]	6.55	6.399	0.147
23	6.99	7.274	−0.287
24	5.00	4.781	0.219
25[a]	6.49	6.399	0.092
26	5.08	5.075	0.004
27[a]	6.63	6.399	0.234
28	7.01	7.274	−0.266

[a] Center point.

3.2. Investigation of in vitro release of *B. pseudocatenulatum* G4 from beads

The release profile of the encapsulated *B. pseudocatenulatum* G4 was compared between alginate–chitosan beads (optimized), non-coated alginate beads (4 g/100 mL) and free cells (as the control). Capsule samples (alginate and alginate + chitosan) were treated with SGF and SIF to control the continuous release characteristics in GIT. The release rate of chitosan–alginate beads was previously studied by regulating pH or adjusting the concentration of alginate in gelling solution (Mi et al., 2002). In the first part of GIT (SGF, pH 1.5), the release amount of Bifidobacterium cells was lower than the capsule samples. After transferring the beads from SGF to SIF, the result showed a larger amount and faster release rate of *B. pseudocatenulatum* G4 cells. The release of *B. pseudocatenulatum* G4 cells reached the maximum level after 3 h. Fig. 3 shows the treatments of

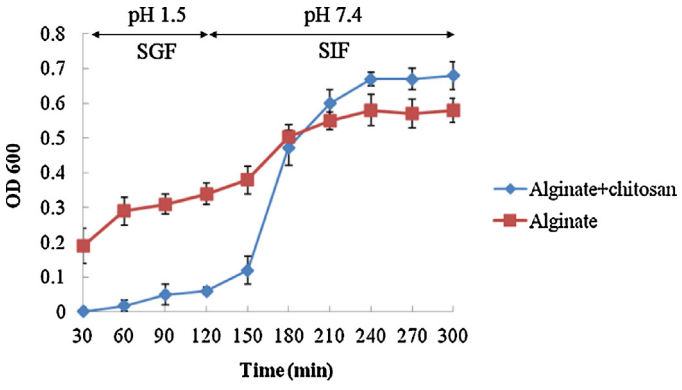

Fig. 3. The effect of simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) on stability of alginate–chitosan and alginate capsules by spectrophotometer.

B. pseudocatenulatum G4 cells in SIF. *B. pseudocatenulatum* G4 cells were released from alginate capsules in both acidic (pH 1.5) and neutral (pH 6.8) conditions. However, the release rate and amount of cells in neutral medium were higher than the release rate in acidic medium. After transferring beads from SGF to SIF, OD value was gradually increased in the first 150 min of incubation. Since, alginate is water soluble anionic, hydrophilic polysaccharide. Its $-COO^-$ groups interacted with Ca^{2+} ions to form "egg-box" structure during the formation of beads in $CaCl_2$ solution. This cross linking activity helps to stabilize the beads. When the beads are treated in SGF, alginate component can undergo acid-catalyzed hydrolysis. In this condition, carboxylate of alginate is neutralized, thus leading to the interruption of electrostatic attraction Ca^{2+} and –COO– groups (Bajpai, Saxena, & Sharma, 2006). SGF media exhibited more acid-catalyzed hydrolysis in non-coated alginate capsules rather than alginate–chitosan capsules.

To sum up, alginate–chitosan capsules were more resistance than alginate capsules and free cells in acidic media. Chávarri et al. (2010) also compared the survival of B. bifidum in the microencapsulated form and free cell. The researchers examined different types of coating material (i.e. alginate, chitosan and quercetin). They also found that the microencapsulation of *B. bifidum* in alginate–chitosan capsules led to improve its survival in simulated gastrointestinal conditions; while the probiotic bacteria microencapsulated with quercetin did not survive during storage at 4 °C. This finding confirms that the efficiency of microencapsulation on the survival of probiotics depends on not only the encapsulation conditions but also the type of coating material. Cook et al. (2011) investigated the suitability of alginate and chitosan microcapsules as an enteric vehicle for delivery of *B. breve* in the simulated gastric juice. The researchers found that coating with chitosan did not let to release *B. breve* cells into simulated intestinal fluid. They showed that the release rate of chitosan-coated *B. breve* cells reduced during exposure to the simulated intestinal fluid. In another survey, Cook et al. (2013) showed that the microencapsulation of *B. breve* significantly improved its survival in low pH (i.e. the gastrointestinal tract). They reported that the chitosan–alginate capsules containing *B. breve* had better resistance than the alginate capsules in the simulated gastric solution. Zhang et al. (2013) also investigated the effect of different microencapsulation methods (i.e. extrusion methods, emulsion methods and coacervation) on the survival of freeze-dried *B. bifidum* as compared to free cells. They found that the encapsulated cells with alginate and chitosan exhibited significantly stronger resistance to artificial gastrointestinal juice than free cells. In fact, the effective encapsulation of *B. pseudocatenulatum* G4 might be due to the deposition of alginate–chitosan multilayers onto alginate matrices (Cook et al., 2013).

The highly porous alginate network found in bacteria-loaded microcapsules might be responsible for limited strength under simulated gastric condition (Allan-Wojtas, Hansen, & Paulson, 2008). Chitosan is commonly used as coating material, because it can induce strong complexes with alginates (Krasaekoopt et al., 2004). It has the protective tolerant against the effects of calcium chelating and anti-gelling agents. In addition, its beads have denser and stronger structure than alginate beads, thereby navigating the breakage and release of the cell(s) (Krasaekoopt et al., 2003; Smidsrød & Skjak-Braek, 1990; Zhou et al., 1998). The beads began to break up rapidly when they were transferred into SIF. As the beads emerged in high pH (6.8), the ion-exchange process between Ca^{2+} and Na^+ ions (from SIF solution) was the main responsible in disintegration of the beads. In this condition, the cell released from beads rapidly. Alginate beads showed faster and easier release than alginate–chitosan beads. As explained by Chávarri et al. (2010), coating of alginate beads with chitosan caused a chitosan–alginate complex. The formation of this complex led to reduce the porosity

Table 5
The count of living cells (log cfu/mL) during incubation of free cells and encapsulated *B. pseudocatenulatum* G4 in GIF.

Time	GIF	Free cells	Alginate	Alginate + Chitosan
0	SGF	8.93 ± 0.4	8.48 ± 0.13	8.72 ± 0.14
30		7.84 ± 0.29	7.38 ± 0.12	8.63 ± 0.48
60		7.13 ± 0.32	7.30 ± 0.20	8.51 ± 0.46
90		6.04 ± 0.16	6.10 ± 0.34	8.24 ± 0.15
120		5.54 ± 0.06	5.81 ± 0.30	7.93 ± 0.28
150	SIF	4.75 ± 0.41	6.04 ± 0.33	7.67 ± 0.58
180		4.53 ± 0.44	5.50 ± 0.05	7.44 ± 0.11
210		4.72 ± 0.57	5.43 ± 0.26	7.46 ± 0.38
240		3.95 ± 0.04	4.93 ± 0.35	6.93 ± 0.30
270		3.90 ± 0.09	4.80 ± 0.03	6.99 ± 0.31
300		3.85 ± 0.70	4.51 ± 0.60	6.53 ± 0.16

GIF: gastro intestinal fluid; SGF: Simulated gastric fluid; SIF: simulated intestinal fluid (SIF).

of alginate beads and decrease the leakage of the encapsulated probiotic. Moreover it was stable at broad pH ranges.

3.3. Evaluation of viability of free cells and entrapped cells of *B. pseudocatenulatum* G4 during incubation in gastrointestinal fluid

Table 5 indicates that the entrapment of *B. pseudocatenulatum* G4 with alginate–chitosan complex significantly ($p \leq 0.05$) protected the cells in high acidic environments. The viability of free cells decreased considerably during incubation. However, the encapsulation of free cells with alginate led to increase the viability of the cell at SGF. In the study, the entrapment of the cells with chitosan and alginate was much more effective than alginate alone to protect *B. pseudocatenulatum* G4. The number of free cells decreased from 8.93 (±0.4) to 5.54 (±0.06) in 120 min incubation at the similar condition to gastric juice; while the bacteria entrapped in alginate showed less decrease as 5.81 in 120 min in SGF. Xaioyan and Xiguang (2009) investigated the effect of microencapsulation on viability of lactic acid bacteria in comparison with its free cells. They found that the immobilization with alginate, gelatin and trehalose additives significantly improved the survival of probiotic cells in the dry culture. They reported that the cells became more stable and the death of the cells encapsulated with alginate, gelatin and trehalose was less intense than alginate alone.

In the present study, chitosan showed a significant ($p \leq 0.05$) effect on the viability of cells. The porosity of alginate allows diffusion of H^+-ions into the gel, hence affecting the cells (Trindade & Grosso, 2000). Hansen, Lan-Wojtas, Jin, and Paulson (2002) also stated that the porosity of alginate matrix increased in the presence of the bacteria during gelation process. It was found that alginate gels had a limited buffering capacity with a highly porous hydrogel structure, providing virtually no barrier. This was affecting the diffusion of H^+-ions into the gel (Le Tien, Millette, Mateescu, & Lacroix, 2004). Bifidobacteria is not as acid tolerant as *L. acidophilus*. The growth of *L. acidophilus* stopped below pH 4.0; while the growth of *Bifidobacterium* spp. is retarded below pH 5.0 (Dave & Shah, 1997). As stated by Smith (1995), food transit time in the stomach depends on the nature of food and it normally remains in the stomach between 2 and 4 h. Therefore, retain of bifidobacteria cells within the encapsulating membrane was evaluated in the exposure of gastric pH within 2 h. When non-coated capsules were treated with SGF, alginate component underwent acid catalyzed hydrolysis. The alginate gel is solubilized via sequestering calcium ions and then entrapped cells are released (Rao, Shiwnarain, & Maharaj, 1989). Champagne, Gaudy, Poncelet, & Neufeld (1992) applied multiple coatings containing poly L-lysine and alginate for microencapsulation purpose. They found that these coating materials reduced the cell release by a factor of 10. Zhou et al. (1997) also showed that the non-coated beads had greater release than chitosan coated beads in acidic fermented milk. This indicates that the chitosan membrane does not significantly decrease the initial release of *Lactococcus lactis* ssp. *cremoris* from the beads.

The growth of lactic acid bacteria is inhibited by chitosan. This is most likely due to linkage of chitosan to the cells (Groboillot, Champagne, Darling, & Poncelet, 1993). This could be the reason for lower acid productivity of the cells coated by chitosan–alginate than the cells coated with alginate alone. It was found that the existence of the membrane as the mass transfer barrier in the system led to reduce the growth rate and acid productivity of the bacteria. After 180 min incubation time, the number of living cells in SIF were 3.85, 4.51 and 6.53 cfu/mL for free cells, alginate encapsulated- and chitosan–alginate encapsulated cells, respectively. The lowest number of living cells was observed during incubation of free cells. This was most likely due to dramatic decrease of the cells during incubation at SGF.

The viability of encapsulated and non-encapsulated cells was compared during incubation at SIF. The results indicated that the viability of all free cells, alginate encapsulated cells and chitosan–alginate encapsulated cells decreased approximately 1.0, 1.5 and 1.5 log (cfu/mL), respectively. Therefore, the encapsulation of the cells appeared to be effective to protect cells during passage through the human stomach. Furthermore, the results suggested that a large number of chitosan–alginate capsules were released in the intestine and 6.53 (±0.16) log (cfu/mL) of cells remained viable after 3 h incubation in SIF. This number was very similar to the viable cells (10^6–10^8) reported by Shah and Ravula (2000). In consequence, much higher numbers of living cells reached to the intestine by encapsulation. High numbers of living probiotic cells (6–8 log cfu/mL) would be essential to have more desirable health benefits (Shah & Ravula, 2000). The results indicated that the viability of *B. pseudocatenulatum* G4 decreased during incubation at SIF especially in the early few hours. This could attribute to sensitivity of *B. pseudocatenulatum* G4 to bile salt. Previous researchers (Picot & Lacroix, 2004) also reported the sensitivity of Bifidobacterium and Lactobacillus to bile salts of human GIT.

4. Conclusion

B. pseudocatenulatum G4 was effectively encapsulated by alginate and coated the beads by chitosan to obtain encapsulated *B. pseudocatenulatum* G4 with a high viability through the human GIT. The application of the highest concentration of alginate (4.4142 g/100 mL) and medium amount of chitosan (0.5578 g/100 mL) resulted in the highest viability of encapsulated *B. pseudocatenulatum* G4. The experimental value certified the predicted value with 95% confidence interval, recommended an appropriate fit between the models and the experimental data. RSM would be an efficient statistical method to determine the optimum conditions for encapsulation *B. pseudocatenulatum* G4 with the desirable viability and release. The current study revealed that *B. pseudocatenulatum* G4 cells had faster and higher release in neutral condition than acidic condition. Furthermore, alginate–chitosan capsule was more resistant than non-coated alginate capsules to SGF. The present study recommends the encapsulation using alginate–chitosan in order to improve the viability and survival of *B. pseudocatenulatum* G4 cells in acidic condition (pH 1.5).

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