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Full Length Research Paper

Survival enhancement of probiotic *Lactobacillus plantarum* CMU-FP002 by granulation and encapsulation techniques

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Two processes of enclosure of probiotic *Lactobacillus plantarum* CMU-FP002, probiotic granules and calcium alginate beads, were studied. Sodium alginate solution at 1.0, 1.5 and 2.0 % (w/v) was used as a binder. The results showed that 20 log cfu/ml initial concentration of cells could be entrapped by the granules and beads with 12 to 13 log cfu/g and 16 cfu/g, respectively. The physical properties of granules and beads revealed that the strength increased when sodium alginate concentration was increased. On the other hand, the dissolution decreased. Probiotic granules completely released the cells within 60 min after being suspended in stimulate gastric fluid (SGF) pH 1.8 and had 2 to 3 log survival cells per gram. Calcium alginate beads, which were formulated from 1.0 and 1.5% (w/v) sodium alginate solution, gradually released bacterial cells and were completely released in SGF within 120 min. The beads formulated from 2.0 %(w/v) sodium alginate solution could not completely release the probiotics. The beads contained more survival cells than granules. Furthermore, the beads formulated from 1.5% (w/v) sodium alginate solution had the highest survival cells (9.30 log cfu/g). Probiotic cells in calcium alginate beads were still high (11 log cfu/g), although they were stored at 4˚C for 5 days alternating with room temperature for 5 days, for a total of 2 month. Further application in broilers will be studied.

Key words: Probiotic, survival enhancement, granulation, encapsulation, *Lactobacillus plantarum*.

INTRODUCTION

Intestinal coccidiosis, caused by various species of *Eimeria,* has become an economically important disease of poultry and livestock throughout the world. The disease can lead to reduced productivity and/or major losses of livestock (Girard et al., 1997). As the world's poultry industry continues to expand, so does the concern for the control of coccidiosis. Jeurissen et al. (1996) reported that the infection of chickens starts after the ingestion of oocysts causing sporozoites to penetrate the epithelium of the villi. After passing through the lamina propria, the sporozoites enter the crypt epithelial cells where they undergo several rounds of asexual and sexual proliferation, thus forming merozoites and later, gametocytes. When macrogametes are fertilized by microgametes, oocysts are formed that are shed in the faeces. For the 7 species of Eimeria, each parasitize inhabits different areas of the intestine. For example, *E. ucervulina* is restricted to the duodenum and *E. tenella* is located in the caeca (Girard et al., 1997). Currently, chemotherapy is used extensively to control the disease, but drug resistance among parasite strains has occurred (Chapman, 1998) and has created interest in the development of alternative control methods. The development and use of probiotics for poultry is based on the knowledge that the gut flora is involved in resistance to enteric infections.

Recently, Kasornpikul et al. (2009 succeeded in

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screening *Lactobacillus plantarum* CMU-FP002 from chicken intestines. This strain indicated probiotic properties such as tolerance to 0.3% bile, growth in widely pH range, utilization of starch, protein and fat. It also had antibacterial reactions to *Salmonella typhimurium* and *Escherichia coli*. *L. plantarum* CMU-FP002 exhibited an 85.63% reduction in the number of *E. tenella* oocysts shed in the faeces compared with the control group. Probiotics *L. plantarum* CMU-FP002 have also been shown to play an important role in protecting broiler from *E. tenella* by several mechanisms during infection, especially by local immunity mechanisms. However, the problems of probiotic application as a food additive in animal feed for livestock are the longevity of probiotic cells and the required properties of probiotics during storage and in the intestinal tract. According to the aforementioned reasons, several animal feed products with added probiotic as food additives have short shelf life. In order to impact the desired health benefits, probiotic bacteria should be contained in the product in higher viable count (7 to 9 log cfu/mL) during their whole product shelf life, which is required to successfully develop foods (Xinhuai et al., 2009).

One of the solutions to these problems is to granulate or encapsulate probiotic. Alginate is one of the materials for encapsulation. It is a natural biopolymer extracted from brown algae. It is composed of linear chains of the α-L-guluronic acid (G) and the β-D-mannuronic acid (M). Alginates form hydrogels in the presence of divalent cations like Ca^{2+} (Bajpai and Sharma, 2004; Pasparakis and Bouropoulos, 2006). Several reports revealed that *Lactobacillus* spp. cells in calcium alginate beads are entrapped by microencapsulation (Chandramouli et al., 2004; Gildas et al., 2009). Besides, some reports revealed that entrapped *L. plantarum* SP 1-3 cell (Ohmomo et al., 2007) and endospores of *Bacillus megaterium* (Chumthong et al., 2008) in granule forms could improve survival rates. Moreover, application of encapsulated probiotic in dry fermented sausages (Muthukumarasamy and Holley, 2006), yoghurt (Kailasapathy, 2006) and ice cream (Homayouni et al., 2008) could improve survival rates during storage and have no significant effect on their sensory properties. Thus, we are interested in enhancing probiotic *L. plantarum* CMU-FP002 living cells using granulation and encapsulation techniques performed by the probiotic granules and calcium alginate beads in this study.

MATERIALS AND METHODS

Materials

Sodium alginate, pepsin from porcine stomach mucosa, pancreatin, trypsin from hog pancreas and bile salts were purchased from Sigma (St. Louis, MO. USA). Calcium chloride and lactose was purchased from Ajax Finechem (New Zealand). Bromocresol purple was purchased from Fisher Scientific (UK). De Man Rogosa Sharpe (MRS) medium was purchased from Labscan (Spain). Bacteriological agar powder was purchased from Himedia (Mumbai, India). All other chemicals were reagent grade.

Bacteria, growth conditions and preparation of cell suspensions

The microorganism used in this study was *L. plantarum* CMU-FP002 from our previous research (Kasornpikul et al., 2009). The bacteria was cultured in MRS broth at 37˚C, checked for purity and maintained on MRS agar (1.5% (w/v) agar in MRS broth). Cells for survival experiments were propagated in MRS broth for 18 to 24 h (stationary phase) at 37˚C. Cells were harvested by centrifugation at 5,000 × g for 10 min at 4˚C, and washed twice with 0.9% (w/v) NaCl. Then, cells were harvested again by centrifugation at 5,000 × g for 10 min at 4˚C. Cell count was determined by pour plate on MRS agar incubate for 48 h at 37˚C. The cell pellet was resuspended in distilled water to 20 log cfu/ml as the initial cell concentration. Cell suspension was stored at 4˚C until formulating granule and calcium alginate beads.

Formulation and evaluation of probiotic *L. plantarum* CMU-FP002 granules

L. plantarum CMU-FP002 granules were prepared using the wet granulation technique. Cell suspension of *L. plantarum* CMU-FP002 with 20 log cfu/ml initial concentration were mixed with the diluent (lactose and corn starch, 1:3% (w/w)). Then, they were gradually added with various concentration of binder (1, 1.5 and 2% (w/v) sodium alginate solution) until the formation of coherent mass. Each step was homogenized by using mortar and pestle mixer. The obtained mass was then passed through a 12-mesh sieve (1.68 mm) and then dried at 40˚C for 24 h. The granules of *L. plantarum* CMU-FP002 were evaluated with regard to physical characteristic, percentage of moisture content and preliminary disintegration using the modified method of Chumthong et al. (2008). Moisture content was determined by moisture analyzer and MX-50 (Jay Instruments and Systems Pvt. Ltd., India). Physical characteristics were determined by using a digital camera (Canon IXUS 860 IS, Japan).

Formulation and evaluation of probiotic *L. plantarum* CMU-FP002 in calcium alginate beads

Sodium alginate was used as a wall material for the microencapsulation of *L. plantarum* CMU-FP002 by extrusion modified from Muthukumarasamy and Holley (2006). Cell suspension of *L. plantarum* CMU-FP002 (in distilled water) at initial concentration 20 log cfu/ml was added at a ratio of 1:5 (v/v) to alginate solution. Encapsule extrusion method was performed by expression of the wall material–culture mixture through a 27G syringe needle drop-wise into $CaCl_2$. The encapsulation parameters such as alginate concentration (1, 1.5, 2 and 2.5% w/v) and calcium chloride concentration (0.1 and 0.5 M) were studied. Hardening time of capsules in calcium chloride was fixed for 30 min at room temperature. Encapsules were held at room temperature for 30 min. Encapsules were separated by filtration through Whatman #4 filter paper. Microcapsules were dried at 40℃ for 24 h before storing at 4℃ until use. Physical characteristics were determined by inverted microscope (Olympus CK2, Japan).

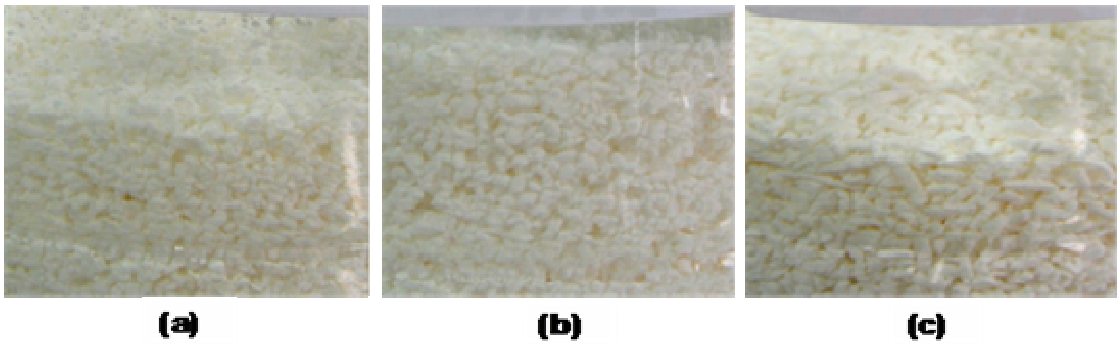

Figure 1. Physical characteristic of *L. plantarum* CMU-FP002 granule using: (a) 1% (w/v) sodium alginate, (b) 1.5% (w/v) sodium alginate and (c) 2% (w/v) sodium alginate. All were examined using digital camera.

Viability assay of cells in granules and calcium alginate beads

Granules and calcium alginate beads, both in dry and wet form, were dissolved in phosphate buffered saline (PBS, pH 7.4). A serial dilution of this suspension was made until a suitable cell density was obtained. The cell suspension was enumerated by pour plate on MRS agar. The plates were then incubated at 37˚C for 48 h. Colonies of bacteria were counted and converted to log cfu (colony forming units). The survival of probiotic cells reported as percentage survival was calculated according to the following equation:

$$\text{Survival (\%)} = \frac{\text{cfu of the dried granules or calcium alginate beads}}{\text{cfu of the wet granules or calcium alginate beads}} \times 100$$

Cell survival of *L. plantarum* CMU-FP002 granules and beads in gastrointestinal tract condition

The procedure for examination was modified from Gildas et al. (2009). Simulated gastric fluid (SGF), consisting of 9 g/L NaCl and 3 g/L pepsin from porcine stomach mucosa, was prepared. Then, pH was adjusted to 1.8 with 0.5 N HCl. The choice of pH 1.8 for SGF took into account the activity of pepsin, which was maximal in a pH range of 1.7 to 3.0 (Tobey et al., 2001). Simulated intestinal fluid (SIF), consisting of 9 g/L NaCl, 10 g/L pancreatin, 10 g/L trypsin from hog pancreas and 3 g/L of bile salts, was prepared. Then, pH was adjusted to 6.5 with 0.1N NaOH. Granules or beads were incubated in SGF for 120 min and subsequently, transferred to SIF for 180 min at 37℃. The control solution was sodium chloride solution (9 g/L) and control samples were free cells, blank granules and blank beads. Granules and beads were dissolved in phosphate buffered saline (PBS) of pH 7.4. Cell counts were determined by serial dilutions in the same buffer and plate counting of MRS agar.

Stability of *L. plantarum* CMU-FP002-loaded alginate particles during storage

A sample of free flowing *L. plantarum* CMU-FP002-loaded alginate particles was tested for stability, which modified the condition from Heidebach et al. (2010). It was stored for 8 weeks, alternating between 4℃ for 5 days and room temperature for 5 days, until the end of the storage period. For predetermined times at week intervals, 1 g of samples was collected and then dissolved and made into serial dilutions in phosphate buffered saline (PBS) of pH 7.4. Cell count was determined by pour plates on MRS agar and incubated for 48 h at 37˚C to determine survival cells of *L. plantarum* CMU-FP002.

Statistical analysis

All data is expressed as means ± SD from at least three independent experiments. Differences between the mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA). The differences were considered significantly different when $p < 0.05$.

RESULTS

Formulation and evaluation of probiotic *L. plantarum* CMU-FP002 granules

Three concentrations of sodium alginate were used to perform granules. The physical appearance of granules is shown in Figure 1. The strength of granules is directly related to the sodium alginate concentration. When sodium alginate concentration was increased, the strength of the granules increased. After drying at 40˚C in hot air oven for 24 h, the granules made from 1 %(w/v) sodium alginate looked like a white short worm line and was easily friable by hand. The properties of formulated granules using 1.5% (w/v) sodium alginate were similar to those using 1% (w/v) except for the white worm lines, which had more length and strength as shown in Figure 1. Among these granulation, 2%(w/v) sodium alginate produced the longest and strongest granules.

Moisture percentages of dried granules, which were produced from 1, 1.5 and 2%(w/v) sodium alginate were 3.04, 3.34 and 3.50%, respectively. The disintegration of obtained granules in distilled water at 37˚C was studied. The granules produced from 1 and 1.5%(w/v) sodium alginate could disintegrate in distilled water at 37˚C, better than granules produced from 2%(w/v).

Disintegration decreased when increasing sodium alginate concentration.

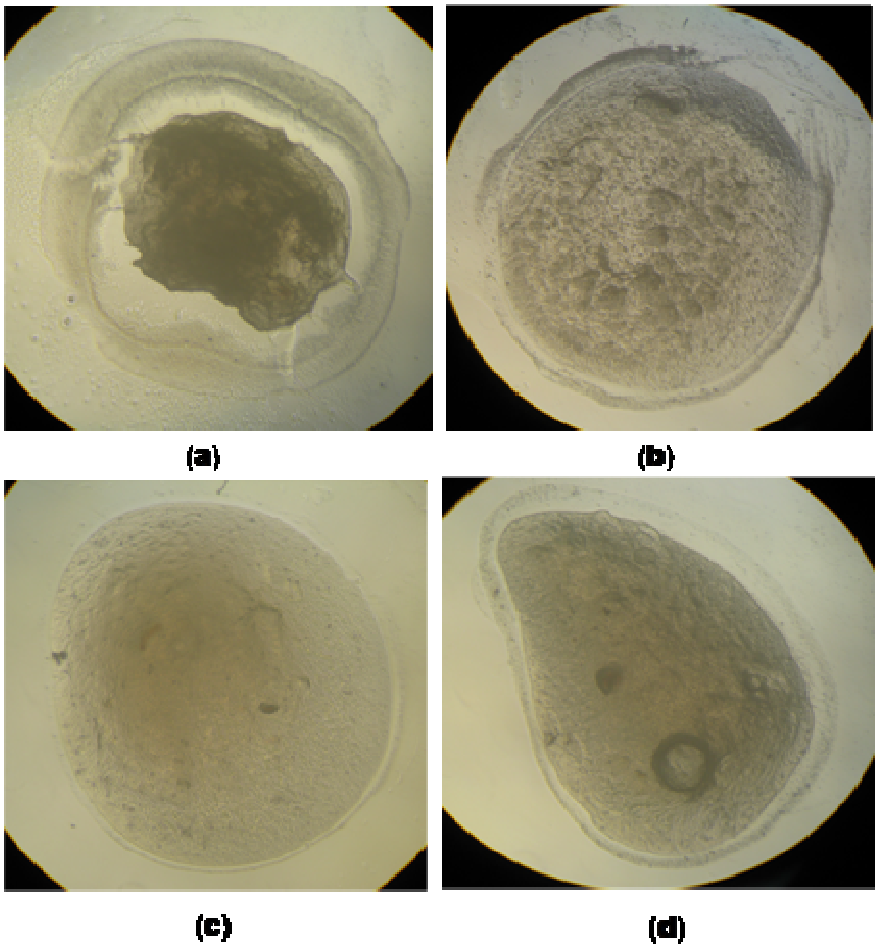

Figure 2. Physical characteristic of *L. plantarum* CMU-FP002 calcium alginate beads, (a)-(d) were prepared by using 1, 1.5, 2 and 2.5% sodium alginate, respectively. All were examined under 40X inverted microscope.

Formulation and evaluation of probiotic *L. plantarum* CMU-FP002 in calcium alginate beads

In this study, the influence of encapsulation parameters such as sodium alginate concentration and calcium chloride concentration were determined and optimized. The physical characteristics of calcium alginate beads were shown in Figure 2. The results showed that increasing sodium alginate concentration improved the beads shape and structural uniformity. Gel bead formation of 1% (w/v) sodium alginate presented a 2 to 3 mm gel bead size, cream-white color, non-spherical, non-smooth and revealed cracks on the surface. When sodium alginate of up to 1.5% (w/v) was used, 2 to 3 mm gel bead size, cream-white color, a nearly spherical shape and a nearly smooth surface ware observed. Cracks on the surface were less observed than using 1% (w/v) sodium alginate. The gel beads made from 2% (w/v) sodium alginate formed 2 to 3 mm gel bead size, cream-white color, spherical shape and smooth surface. Using 2.5% (w/v) sodium alginate, gel beads with a similar property to 2% (w/v) was observed except its spheroid shape. Gel beads, which performed in 0.5 M calcium chloride, had a higher strength than in 0.1 M calcium chloride.

For preliminary tests, dissolution of obtained blank beads in stimulated gastric juice (SGF) pH 1.8 was studied. The beads formed in 0.1 M calcium chloride could dissolve in SGF pH 1.8 better than blank beads formed in 0.5 M calcium chloride. In this experiment, low dissolution of blank beads made from 2.5% (w/v) sodium alginate and 0.5 M calcium chloride was observed. Thus, three sodium alginate concentrations, 1, 1.5 and 2% (w/v) with 0.1 M calcium chloride, were used to form the gel beads in the next experiment.

Survival of *L. plantarum* CMU-FP002 cell in granules and calcium alginate beads

Table 1 shows the viability and survival percentages of *L. plantarum* CMU-FP002 cells in granules and calcium

Table 1. Viable cell and survival percentages in wet granules, dried granules, wet calcium alginate beads and dried calcium alginate beads, which made from various concentration of sodium alginate (1, 1.5 and 2% w/v).

Sodium alginate (%w/v)	Viable cell (log cfu/g)				Survival (%)	
	Wet granules	Wet beads	Dried granules	Dried beads	Granules	Beads
1.0	18.74 ± 0.02^{b}	19.20 ± 0.02^{a}	12.95 ± 0.04^{h}	16.11 ± 0.03^{e}	69.10^{B}	83.91^{A}
1.5	18.36 ± 0.10^{c}	19.37 ± 0.01^{a}	13.27 ± 0.02^{g}	16.33 ± 0.06^{de}	72.28^{C}	84.31^{A}
2.0	18.46 ± 0.08^{bc}	19.41 ± 0.03^{a}	14.36 ± 0.02^{f}	16.37 ± 0.06^{d}	77.79^{D}	84.33^{A}

* Each value is the mean ± SD of three trials. The small alphabet indicate that the values are significantly different in viable cell amount among wet granules, wet beads, dried granules and dried beads treatment ($p < 0.05$, n = 3). The big alphabet indicate that the values are significantly different in survival percentages between granules and beads treatment ($p < 0.05$, n = 3).

alginate beads before and after being dried at 40˚C in a hot air oven for 24 h. Approximate 4 to 5 log cfu/g of *L. plantarum* CMU-FP002 cells in wet granules were significantly decreased after they were dried at 40˚C ($p < 0.05$). Similarly, cell survival in wet beads (~19 log cfu/g) were significantly higher than those in dried beads (~16 log cfu/g) ($p < 0.05$). Approximate 19 log cfu/g of *L. plantarum* CMU-FP002 cells in wet calcium alginate beads were achieved. Cell survival in wet calcium alginate beads was significantly higher in wet granules (~18 log cfu/g) ($p < 0.05$). After being dried at 40˚C, survival cells of dried beads were significantly higher than dried granules ($p < 0.05$). The results showed that live cells of calcium alginate beads, produced from 2% (w/v) sodium alginate, were the highest ($p < 0.05$). On the other hand, viable cells of dried granules, formed from 1% (w/v) sodium alginate, were the lowest ($p < 0.05$).

The results showed that increasing sodium alginate concentration, significantly increased the viability when it was in dried granules and beads ($p < 0.05$), but it was not achieved in wet granules and beads.

Survival percentages of dried granules and beads were calculated from viable cells in wet granules and dried granules and wet beads and dried beads, respectively. The results revealed that the survival percentages of the beads were higher than granules ($p < 0.05$). Furthermore, the result showed that survival percentages of both granules and beads increased with increased in sodium alginate concentration.

Cell survival of *L. plantarum* CMU-FP002 granules and beads in gastrointestinal tract condition

In order to evaluate the release and viability of *L. plantarum* CMU-FP002 granules and encapsules in gastrointestinal condition, *L. plantarum* CMU-FP002 granules and encapsules were tested in simulated gastric fluid (SGF) for 120 min and simulated intestinal fluid (SIF) for 180 min. The effect of sodium alginate concentrations on the viability of *L. plantarum* CMU-FP002 under SGF and SIF is shown in Figure 3. Three formulas of granules were disintegrated within 60 min in SGF (Line A-C). Moreover, all calcium alginate bead formulas were disintegrated with more difficulty than all granule formulas (Line D-F). Granules and gel beads produced from higher concentration of sodium alginate were more difficult to disintegrate. Gel beads made from 2% (w/v) sodium alginate did not completely dissolve (Line F). A viable count of *L. plantarum* CMU-FP002 in the granules decreased rapidly with increasing contact time in SGF (from ~ 12 - 14 to 8 - 9 log cfu/g) (Line A-C) and SIF (from ~ 8 - 9 to 2-3 log cfu/g) (Line A-C). On the other hand, the survival of cells in gel beads gradually declined with increasing contact time in SGF and SIF (from ~ 15 log to 6 - 9 log cfu/g) (Line D-F). However, in entrapped bacteria, both granules and beads survived well in gastrointestinal conditions compared to non-entrapped free bacterial cells. Moreover, the results revealed that the viability of encapsulated bacteria in gastrointestinal conditions increased with an increasing alginate gel concentration of 1 to 1.5% (w/v) as shown in line D and E. The higher the concentration of sodium alginate, the better the protection of bacterial cells was achieved. However, in 2% (w/v) of sodium alginate preparation, dissolution did not complete (Line F).

Stability of *L. plantarum* CMU-FP002-loaded alginate particles during storage

In this experiment, it was found that encapsulation technique could protect higher number of live cells. Therefore, probiotic encapsules, which were made from 1.5% sodium alginate were selected for storage study. The stability of *L. plantarum* CMU-FP002 entrapped in alginate gel beads during alternating storage between 4℃ for 5 days and room temperature for 5 days is shown in Figure 4. The survival of *L. plantarum* CMU-FP002 loaded in alginate beads gradually declined during storage. The survival was maintained at approximately ~11 log cfu/g after storage for 8 weeks.

DISCUSSION

Wet granulation, the process of adding a liquid solution to powders is one of the most common ways to granulate.

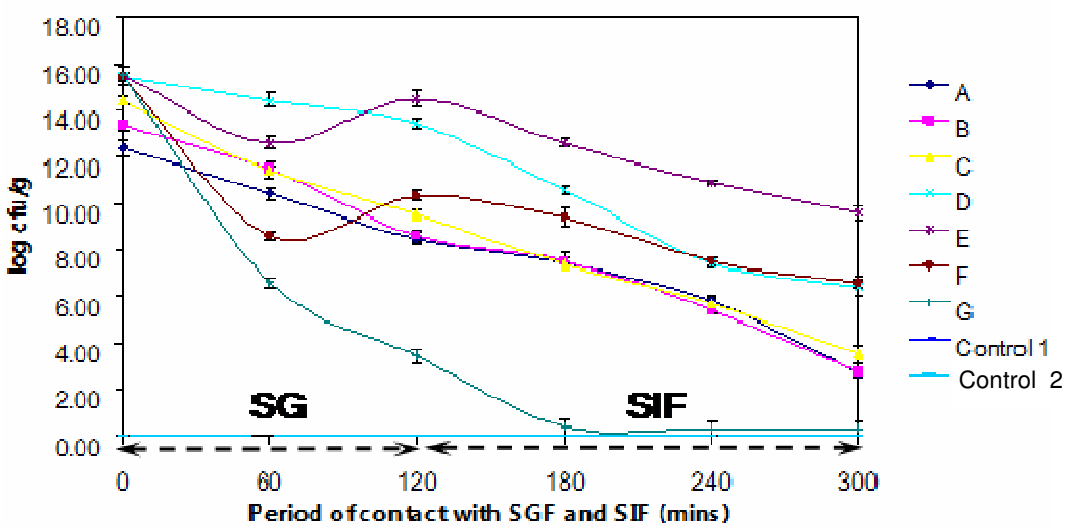

Figure 3. Viable cell of granules and calcium alginate beads formula in contact with SGF for 120 min and SIF for 180 min. *L. plantarum* CMU-FP002 granules, A-C were prepared by using 1, 1.5 and 2% sodium alginate, respectively. *L. plantarum* CMU-FP002 calcium alginate beads, D-F were prepared by using 1, 1.5 and 2% sodium alginate, respectively. G : free *L. plantarum* CMU-FP002 cells, Control 1 : blank granules, Control 2 : blank beads.

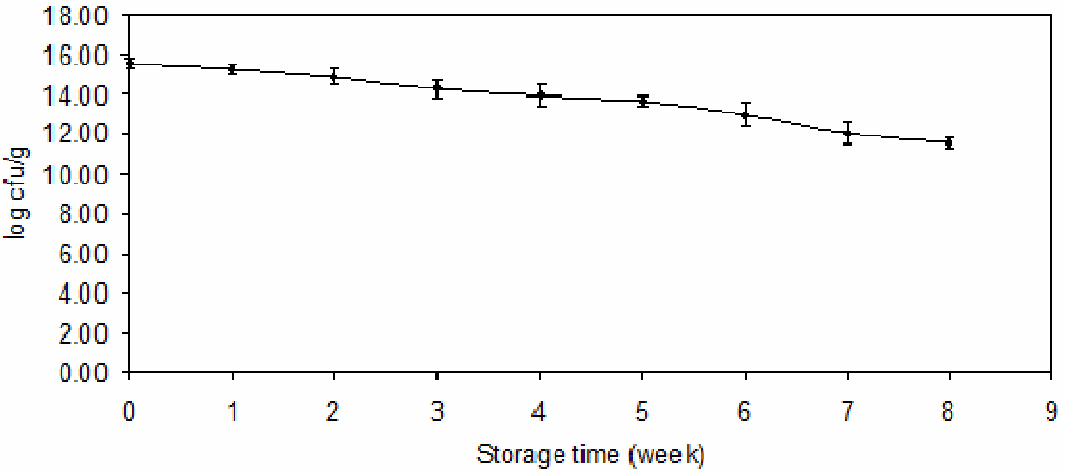

Figure 4. Viability of *L. plantarum* CMU-FP002 in calcium alginate beads during storage for 8 week. Each value is the mean ± SD of three trials.

In this study, the diluents were lactose and corn starch, 1:3% (w/w), with sodium alginate as a binder. Lindberg and Jönsson (1985) reported that lactose 100 mesh was granulated faster than the 350 mesh quality. Corn starch required large volumes of granulating solution. Consequently, a suitable ratio for the end-point determination when granulating a mixture of lactose 100 mesh and corn starch was 2:1 ratio, with povidone as a binder. Sodium alginate solution mixed into the powders can form bonds between powder particles that are strong enough to lock them together (Michael, 2002). The density of each granule increased by increasing the amount of binding solution. Therefore, controlling the quantity of binder was one factor to control the density and strength of the granules (Michael, 2002). In this study, results revealed that increasing the strength and length of granules depended on more viscosity of solutions that was caused by increase in the sodium alginate concentration. Similarly, Chumthong et al. (2008) reported that sodium alginate acted as a viscosifier in the granules formulation, which may be attributed to the bio-adhesive property. Moisture content in dried granules trends to increase with increase in alginate concentration. It may be caused by more granule strength not allowing

the water to evaporate into the atmosphere.

The formation of calcium alginate beads may be related to several factors. In this study, calcium alginate beads were formulated from 1, 1.5, 2 and 2.5% (w/v) sodium alginate solution with 0.1 and 0.5M $CaCl_2$. In the solution, alginates behave like flexible coils. The gel formation of alginate is mainly achieved by the exchange of sodium ions with divalent cations such as Ca^{2+}, Mn^{2+}, Zn^{2+}, or Cu^{2+}. However, depending on the interaction with divalent metal ions (such as Ca^{2+}), they form an ordered structure (Ana et al., 1999). In this study, the beads, which were formed in 0.5M $CaCl_2$, had more strength than in 0.1M $CaCl_2$, it might be explained based on the number of Ca^{2+} ions. As a result, in the pre-dissolution, stronger blank beads could not dissolve in SGF solution. Structural and mechanical properties of calcium-alginate capsules can also be tuned by adjusting the concentration of sodium alginate or calcium chloride. Low solubility calcium salts reduce the gelation rate and increase both the structural uniformity and the mechanical strength of gels (Thu et al., 1996; Wang and Spencer, 1998). Our result showed that 2.5% (w/v) alginate concentration, both in 0.1 and 0.5M $CaCl_2$, had difficult in forming spherical shapes because of their high viscosity. Similarly, Ariel et al. (2006) reported that maximum encapsulation efficiency was achieved by using higher molecular weight alginate and increasing the alginate concentration. Furtheremore, Chandramouli et al. (2004) reported that 2% (w/v) alginate solution had difficulty in forming spherical shapes. From our results, the higher concentration of sodium alginate and the better protection of bacterial cells were achieved. Increasing the biopolymer (sodium alginate) concentration may increase the number of binding sites for Ca^{2+} ions. As a result, a more densely cross-linked gel structure was formed.

Survival percentage of *L. plantarum* CMU-FP002 in dried gel beads was higher than in dried granules. This might be attributed to the interaction of gel formation causing stronger and denser structures than the interaction of granulation. In addition, granule structure had non-smooth surfaces and many cracks on the surface. According to these reasons, during the drying process, heat could contact with the cells in granules more than the cells in gel beads. Therefore, cells, which were entrapped in calcium alginate bead forms, were protected from heat, better than cells in granule forms. However, live cells in dried granules (~ 13 to 14 log cfu/g) in our experiment were still high enough for health benefits.

For the viability of *L. plantarum* CMU-FP002 granules and beads in SGF and SIF, results of our study corresponded well with Chandramouli et al. (2004), Lee and Heo (2000) and Ross et al. (2006) who indicated that *L. acidophilus* CSCC 2400, *Bifidobacterium longum* and *Bifidobacterium infantis* in calcium alginate spheres survived when exposed to SGF. Furthermore, Chandramouli et al. (2004) reported that the viability of encapsulated *L. acidophilus* CSCC 2400 in simulated gastric conditions increased when increasing alginate gel concentration from 0.75% to 1.8% (w/v). Cui et al. (2000) indicated that when the bifidobacteria was immobilized with alginate or even poly-*l*-lysine treatment, the survival of bifidobacteria was highly enhanced in the low pH conditions. Ross et al. (2006) reported that microscopic examination of the microcapsules showed that the bacteria remained entrapped within the capsule material in SGF and were released when transferred to SIF. It seemed likely that in our experiment, gastric fluid entered the microparticles through the surface pinholes resulting in a loss of viability. Thus, the survival of *L. plantarum* CMU-FP002 declined as the incubation time increased due to the detrimental effects of low pH on their cells. However, the dense membrane was expected to create diffusion resistance through the beads, which resulted in lower diffusion of SGF and SIF. Consequently, cell survival increased with increased alginate gel concentration. The strengthening of the surface membrane and the size of microparticles offered other choices for further enhancement of gastric resistance (Cui et al., 2000). Granulation technique has few reports regarding the application of entrapped bacterial cells. This might be as a result of the less protection of granulation when contacting SGF. Similarly, Al-Mohizea et al. (2007) reported that the wet granulation technique was not favorable for producing the yeast tablets due to the problems of color darkening and the reduction of the fermentation power of the yeast as a result of the early start of the fermentation process due to the presence of moisture.

The stability of bacterial cells entrapped in calcium alginate particles improved after encapsulation with alginate when compared to free *L. plantarum* CMU-FP002 cultures. The effect of storage conditions on viability of *L. plantarum* CMU-FP002 in calcium alginate beads was similar to Cui et al. (2000) who found that the stability of free flowing bifidobacteria-loaded alginate poly-*l*-lysine microparticles was significantly improved during storage at 4°C in a refrigerator when compared to bifidobacteria cultures. Our results indicated that encapsulated *L. plantarum* CMU-FP002 cells in calcium alginate beads could improve the viability of bacterial cells, although it was kept in an alternating condition between 4˚C for 5 days and room temperature for 5 days.

Conclusions

In summary, both granulation and encapsulation techniques were successful for entrapping *L. plantarum* CMU-FP002 with high survival cells enough for the use as a feed additive in broilers. However, the improvement of granulation technique is required to enhance the

retention efficiency during contact with SGF. The optimum conditions of microencapsulation preparation in this paper were 1.5% sodium alginate with 0.1 M calcium chloride and 30 min hardening time at room temperature. This condition was proved to be efficient in increasing the viability of probiotic bacteria in SGF compared to non-encapsulated free cells. The number of viable probiotic bacteria in gel beads, made from 1.5% sodium alginate, was 11 log cfu/g at the end of 8 weeks of storage. The results of this study also indicated that alginate microcapsules could be used as a protective delivery vehicle for administering viable probiotic bacteria. Further studies are needed for practical application in broilers.

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REFERENCES

Al-Mohizea AM, Ahmed MO, Al-jenoobi FI, Mahrous GM, Abdel-Rahman AA (2007). Formulation and evaluation of dried yeast tablets using different techniques. Eur. J. Pharm. Biopharm., 67: 253-259.

Ana B, Mannuel M, Domingo C (1999). Formation of calcium alginate gel capsules: Influence of sodium alginate and $CaCl_2$ concentration on gelation kinetics. J. Biosci. Bioeng., 88: 686-689.

Ariel CWJ, Isabelle M, Todd B, Ronald JN (2006). Granulation of subtilisin by internal gelation of alginate microspheres for application in detergent formulation. Enzyme Microb. Tech., 38: 265-272.

Bajpai SK, Sharma S (2004). Investigation of swelling/degradation behavior of alginate beads crosslinked with Ca^{2+} and Ba^{2+} ions. React. Funct. Polym., 59: 129-140.

Chandramouli V, Kailasapathy K, Peiris P, Jonesb M (2004). An improved method of microencapsulation and its evaluation to protect *Lactobacillus* spp. in simulated gastric conditions. J. Microbiol. Meth., 56: 27-35.

Chapman HD (1998). Evaluation of the efficacy of anticoccidial drugs against *Eimeria* species in the fowl. Int. J. Parasitol., 28: 1141-1144.

Chumthong A, Kanjanamaneesathian M, Pengnoo A, Wiwattanapatapee R (2008). Water-soluble granules containing *Bacillus megaterium* for biological control of rice sheath blight: formulation, bacterial viability and efficacy testing. World J. Microb. Biot., 24: 2499-2507.

Cui J-H, Goh J-S, Kim P-H, Choi S-H, Lee B-J (2000). Survival and stability of bifidobacteria loaded in alginate poly-*l*-lysine microparticles. Int. J. Pharm., 210: 51-59.

Gildas GK, Thierry V, Saïd E, Eric M (2009). Microencapsulation of *Lactobacillus plantarum* spp. in an alginate matrix coated with whey proteins. Int. J. Food Microbiol., 129: 103-105.

Girard F, Fort G, Yvore P, Quere P (1997). Kinetics of specific immunoglobulin A, M and G production in the duodenal and caecal mucosa of chickens infected with *Eimeria acervulina* or *Eimeria tenella*. Int. J. Parasitol., 27: 803-809.

Heidebach T, Först P, Kulozik U (2010). Influence of casein-based microencapsulation on freeze-drying and storage of probiotic cells. J. Food Eng., 98: 309-316.

Homayouni A, Azizi A, Ehsani MR, Yarmand MS, Razavi SH (2008). Effect of microencapsulation and resistant starch on the probiotic survival and sensory properties of synbiotic ice cream. Food Chem., 111: 50-55.

Jeurissen SHM, Janse EM, Vermeulen AN, Vervelde L (1996). *Eimeria tenella* infections in chickens: Aspects of host-parasite: Interaction. Vet. Immunol. Immunop., 54: 231-238.

Kailasapathy K (2006). Survival of free and encapsulated probiotic bacteria and their effect on the sensory properties of yoghurt. LWT., 39: 1221-1227.

Kasornpikul C, Chaiyasut C, Sirithanyalug B, Aeangwanich W, Pewnim T (2009). Effect of probiotic *Lactobacillus plantarum* CMU-FP002 on oocyst shedding by broilers inoculated with *Eimeria tenella*. Avian Biol. Res., 2: 157-159.

Lee KY, Heo TR (2000). Survival of *Bifidobacterium longum* immobilized in calcium alginate beads in simulated gastric juices and bile salt solution. Appl. Environ. Microbiol., 66: 869-873.

Lindberg NO, Jönsson C (1985). The granulation of lactose and starch in a recording high-speed mixer, Diosna P25. Drug Dev. Ind. Pharm., 11: 387-403.

Michael TD (2002). The granulation process 101: basic technologies for tablet making. Pharm. Technol., pp. 8-12.

Muthukumarasamy P, Holley RA (2006). Microbiological and sensory quality of dry fermented sausages containing alginate-microencapsulated *Lactobacillus reuteri*. Int. J. Food Microbiol., 111: 164-169.

Ohmomo S, Nitisinprasert S, Kraykaw D, Pholsen P, Tanomwongwattana S, Tanaka O, Tomoyuki S, Takehiro N (2007). Attempt to practical use of *Lactobacillus plantarum* SP 1-3 in spray dried granule form for making good quality silage in Thailand. Kasetsart J. Nat. Sci., 41: 34-42.

Pasparakis G, Bouropoulos N (2006). Swelling studies and *in vitro* release of verapamil from calcium alginate and calcium alginate–chitosan beads. Int. J. Pharm., 323: 34-42.

Ross C, Rangika W, Luz S, MaryAnn A (2006). Synbiotic microcapsules that enhance microbial viability during nonrefrigerated storage and gastrointestinal transit. Appl. Environ. Microbiol., 72: 2280-2282.

Thu B, Bruheim P, Espevik T, SmidsrPrd O, Soon-Shiong P, Skak-Braek G (1996). Alginate polycation microcapsules II. Some functional properties. Biomaterials, 17: 1069-1079.

Tobey NA, Hosseini SS, Caymaz-Bor C, Wyatt HR, Orlando GS, Orlando RC (2001). The role of pepsin in acid injury to esophageal epithelium. Am. J. Gastroenterol., 96: 3062-3070.

Wang X, Spencer HG (1998). Calcium alginate gels: formation and stability in the presence of an inert electrolyte. Polymer, 39: 2759-2764.

Xinhuai Z, Yu Z, Dan L (2009). Elimination of acidic or oxidative stress for four probiotics with some chemicals *in vitro*. Afr. J. Microbiol. Res., 3: 353-357.