CHAPTER 4
RESULTS AND DISCUSSION

4.1 Moisture content and physical properties of QF- and SF-FDTB

In this study, the QF- and SF-FDTB from three commercial brands (Golden Chef®, Special Sacoo®, and Thaiworld®) were used as supporting material for immobilization of *B. longum, B. bifidum*, and *B. infantis* The TSB were white and hard beads with the diameter ca 2.1 mm. The completely gelatinized beads were transparent and had soft texture.

Two freezing methods, slow and quick freezing, were used to modify the pore size and porosity of gelatinized beads from three commercial brands. After drying, moisture content and the physical properties (bulk volume, diameter, microstructure, porosity, specific surface area, water-holding capacity, adsorption capacity, adsorption behavior, and gel strength of reformative beads) of QF- and SF-FDTB from three commercial brands were determined and showed the results in Figures 2-21 and Tables 1-2, respectively. Each bar in Figures 2-19, 21 and each point in Figure 20 represent the mean from 3 replicates. Results with different letters are significantly different ($p \leq 0.05$ by Duncan's multiple range test). Standard error bars are included.

Moisture content of the gelatinized TSB was statistically significant ($p \leq 0.05$) between all tested brands and the values for Golden Chef®, Special Sacoo® and Thaiworld® were 91.97±0.11, 92.26±0.12, and 92.59±0.13%, respectively (Figure 2). The shape of the gelatinized beads were similar and generally spherical. Dry weight of 100 beads from two freezing methods and three commercial brands were not significant difference ($P>0.05$) with the average of 0.56 g per 100 beads (Figure 3). QF- and SF-FDTB from three

commercial brands had either spherical or elliptical shape, resulting from the compaction of gelatinized beads during freezing. After freeze-drying, the moisture content of SF- and QF-FDTB from three commercial brands were not significant difference (P>0.05) with the average of 4.89% (Figure 4). The water-holding capacity of QF- and SF-FDTB for all treatments were not difference (P>0.05) with the average of 9.46 per 100 beads (Figure 4).

Size of QF- and SF-FDTB could be determined by using micrometer measurement and bulk volume measurement. However, the diameter showed similar values for all treatments except the Special Sacoo®SF-FDTB (Figure 5). Whereas the bulk volume of 100-QF- and SF-FDTB showed significant difference ($p \leq 0.05$) of commercial brand or freezing method (Table 1 and Figures 6-7). The SF-FDTB had more bulk volume than the QF-FDTB ($p \leq 0.05$) (Figure 6) and Thaiworld® had the highest ($p \leq 0.05$) value of bulk volume (Figure 7). The different surface property of SF-FDTB and QF-FDTB from Golden Chef®, Special Sacoo®, and Thaiworld® were detected (Figures 8, 9). The SF-FDTB had a puffy surface with large open pore, whereas the QF-FDTB had smooth and some part of closed surface area. Measurement with micrometer may compress the soft puffy surface of SF-FDTB, resulted in lower diameter detection. In contrast, the puffy surface may increase the bulk volume of SF-FDTB.

The porosities of QF- and SF-FDTB from Golden Chef®, Special Sacoo®, and Thaiworld® were calculated from the true volume and granular volume of QF- and SF-FDTB. True volume of 100 beads from two freezing methods and three commercial brands were not significant difference (P>0.05) with the average of 0.308 cm^3 (Figure 10). Whereas the QF-FDTB had more granular volume than the SF-FDTB ($p \leq 0.05$) (Figure 13) and the samples from Thaiworld® had the highest ($p \leq 0.05$) value of granular volume (Figure 14). The porosity of QF-FDTB was significantly higher than that of SF-FDTB ($p \leq 0.05$) (Figure 15). The porosities of QF-FDTB and SF-FDTB were

90.81±0.71, and 89.23±0.87%, respectively. The Golden Chef® had higher specific surface area when compared with Special Sacoo®, and Thaiworld® for both QF-FDTB and SF-FDTB (Figure 16).

The SF-FDTB provided the porous bead with the large pore size (Figure 17) with the average diameter of 57.04 μm whereas the QF-FDTB provided the beads with the smaller pore size (Figure 18) with the average diameter of 11.18 μm for all commercial brands. A possible reason for this observation is that during slow freezing, ice crystals could grow both in inter-granular and intra-granular spaces. Ice crystals have a lower water vapor pressure than regions within the granular, and water therefore moves from the granular to the growing crystals, resulting in deforming of the structure of the beads. In addition, the dimension of soft gelatinized beads could be expanded by the volume of ice after freezing. Quick freezing causes the granular surface to form a crust and prevents further expansion and smaller ice crystals form within intra-granular spaces that reduce damage to the structure of the bead. During freeze-drying process, the ice sublimes directly to vapor without melting (Fellow, 2000). When the beads were completely dried, the porous beads were obtained and pore size of the FDTB depended on the size of ice crystal. The porosity of QF-FDTB was higher than that of SF-FDTB and the results agreed with the experiment of Shan-Yang *et al.* (1999). Structure modification of gel beads by using freeze-drying in order to produce the porous structure was also reported by Tal *et al.* (1997) and Whitehead *et al.* (2000) who produced the porous alginate beads. Fwu-Long *et al.* (2002) produced freeze-dried chitosan beads.

The dimension of bifidobacterial cells was 0.5-1.5 x 1.5-8 μm (Tamime and Robinson, 2000). Comparison of the dimension of bifidobacterial cells and the pore size of QF- and SF-FDTB indicates that the bifidobacterial cells could easily pass through the porous structure of QF- and SF-FDTB. However, bifidobacteria are non-motile (Ballongue, 1998), then it is necessary

to use the water adsorption mechanism of QF- and SF-FDTB to adsorb bifidobacterial cells into the beads.

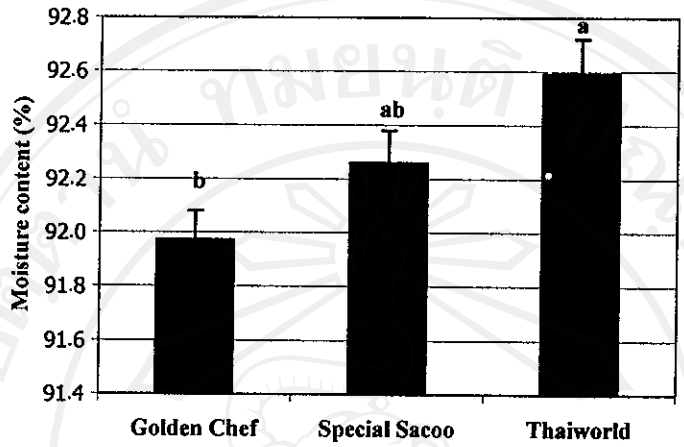

Figure 2 Moisture content of gelatinized TSB from Golden Chef®, Special Sacoo®, and Thaiworld®.

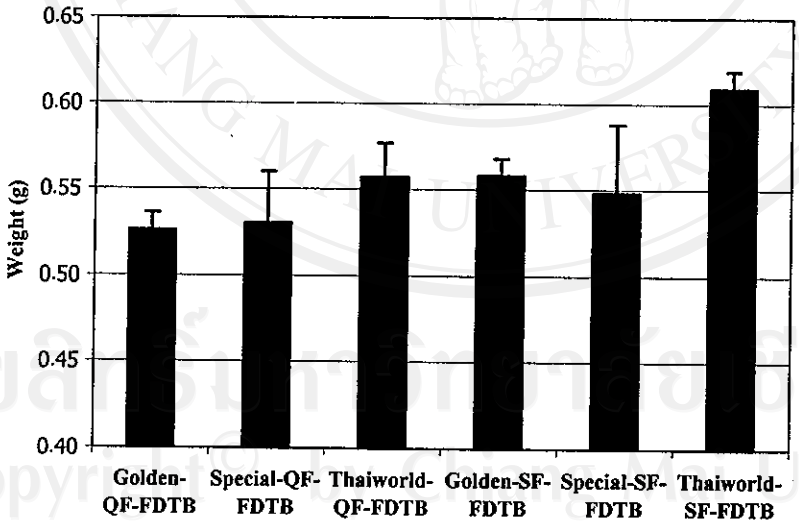

Figure 3 Effects of commercial brands and freezing methods on the weight of 100-FDTB.

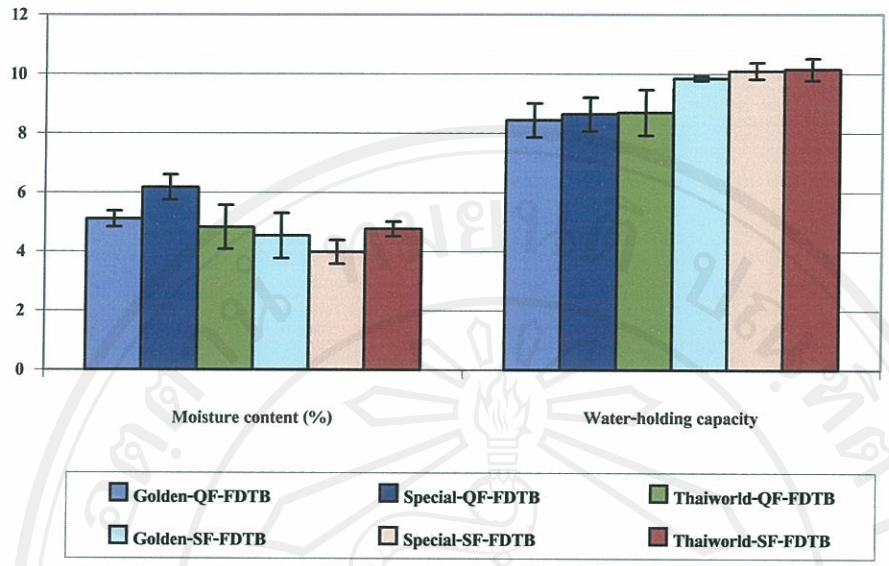

Figure 4 Effects of commercial brands and freezing methods on the moisture content and water-holding capacity of 100-FDTB.

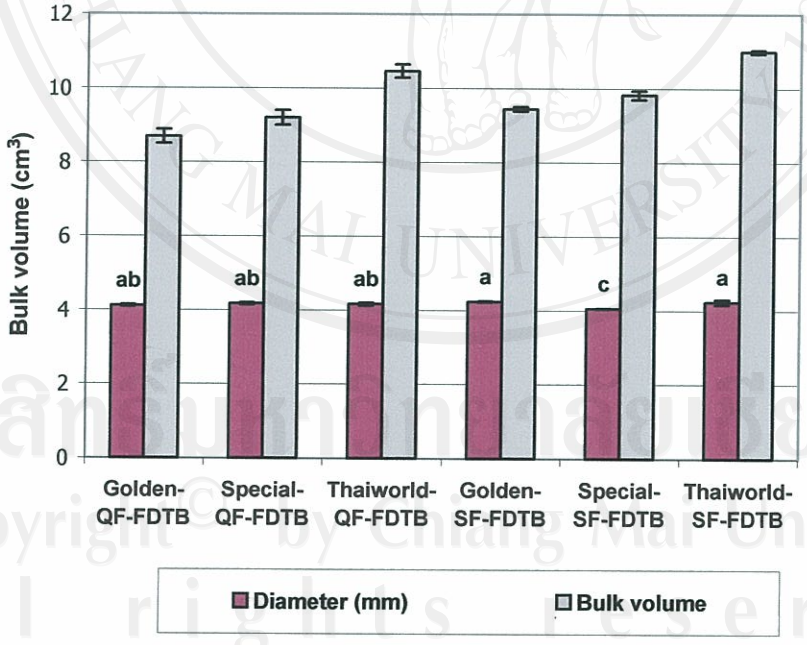

Figure 5 Effects of commercial brands and freezing methods on the diameter and bulk volume of 100-FDTB.

Table 1 Analysis of variance of effects of commercial brands and freezing methods on the diameter and bulk volume of 100-FDTB.

Source of variance	df	Bulk volume	
		MS	p
Freezing method (A)	1	1.82	0.00*
Brand (B)	2	4.41	0.00*
AB	2	0.02	0.76
Error	12	0.06	

* significant ($p \leq 0.05$)

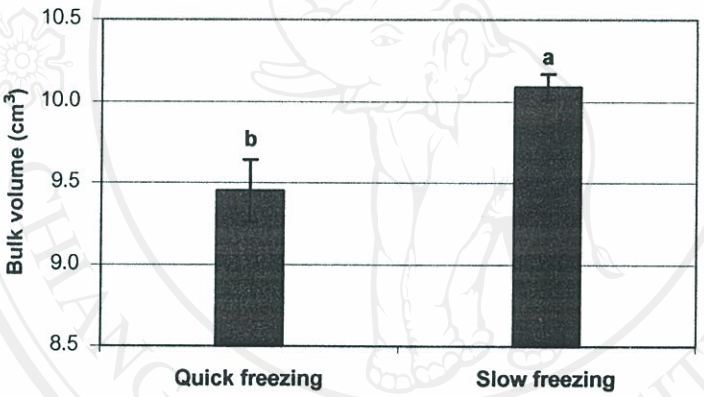

Figure 6 Effect of freezing methods on the bulk volume of 100-FDTB.

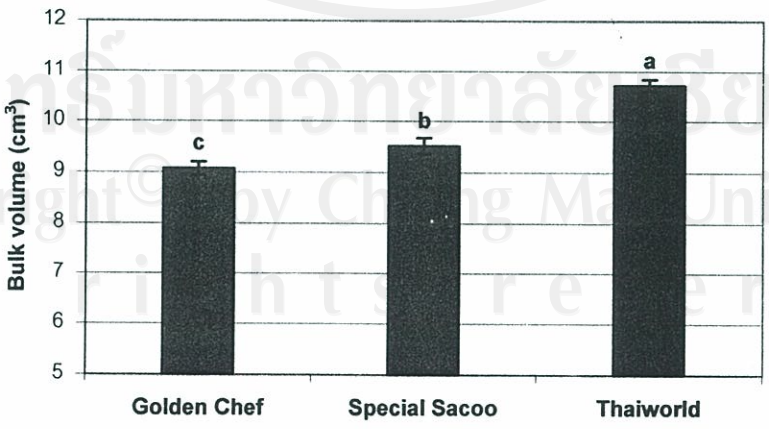

Figure 7 Effect of commercial brands on the bulk volume of 100-FDTB.

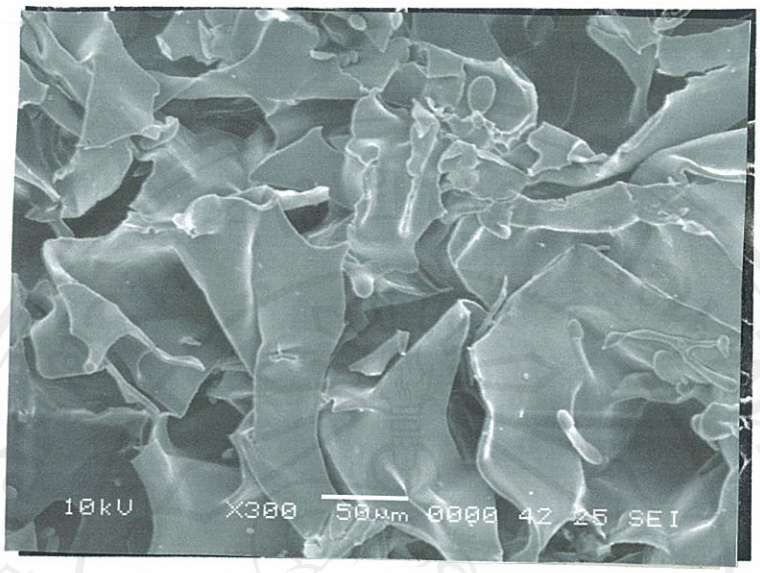

Figure 8 Scanning electron micrograph of the outer surface of SF-FDTB at magnification x 300.

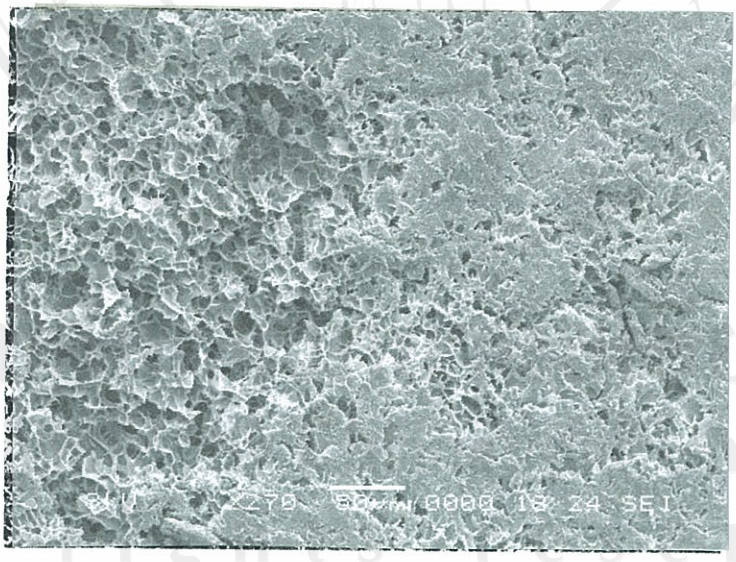

Figure 9 Scanning electron micrograph of the outer surface of QF-FDTB at magnification x 270.

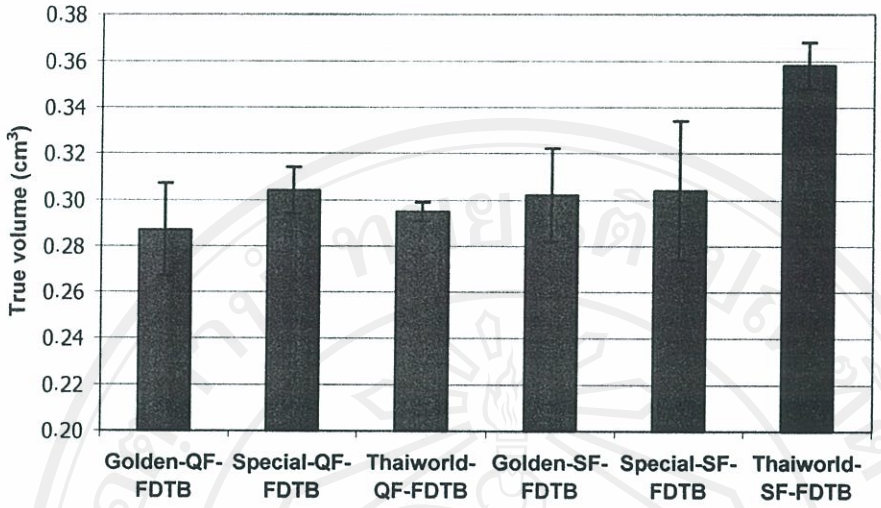

Figure 10 Effects of commercial brands and freezing methods on the true volume of FDTB.

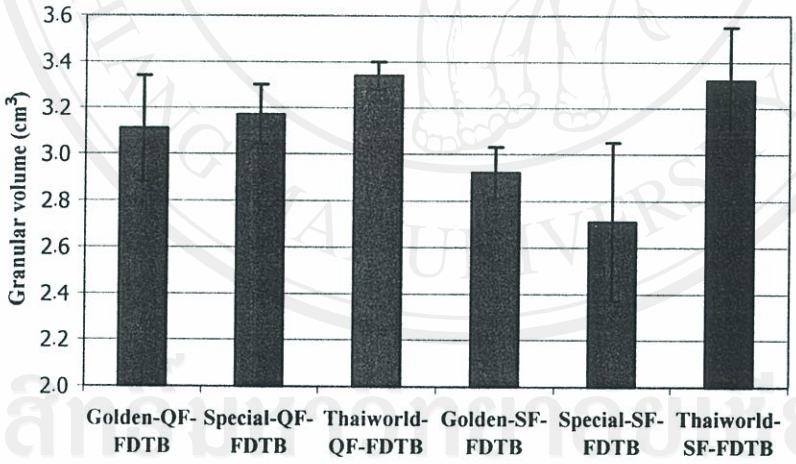

Figure 11 Effects of commercial brands and freezing methods on the granular volume of FDTB.

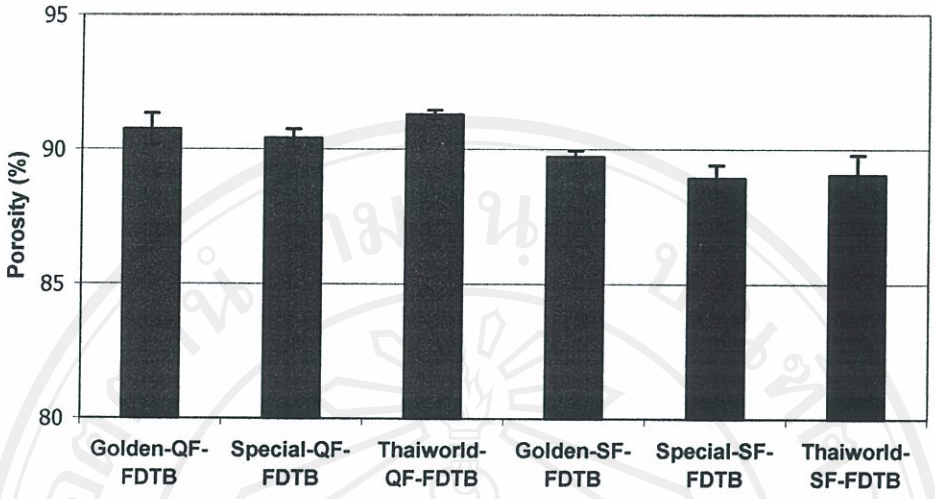

Figure 12 Effects of commercial brands and freezing methods on the porosity of FDTB.

Table 2 Analysis of variance of effects of commercial brands and freezing methods on the granular volume and porosity of FDTB.

Source of variance	df	Granular volume		Porosity	
		MS	p	MS	p
Freezing method(A)	1	0.23	0.04*	11.17	0.00*
Brand(B)	2	0.26	0.01*	0.56	0.45
AB	2	0.07	0.22	0.56	0.45
Error	12	0.04		0.66	

* significant ($p \leq 0.05$)

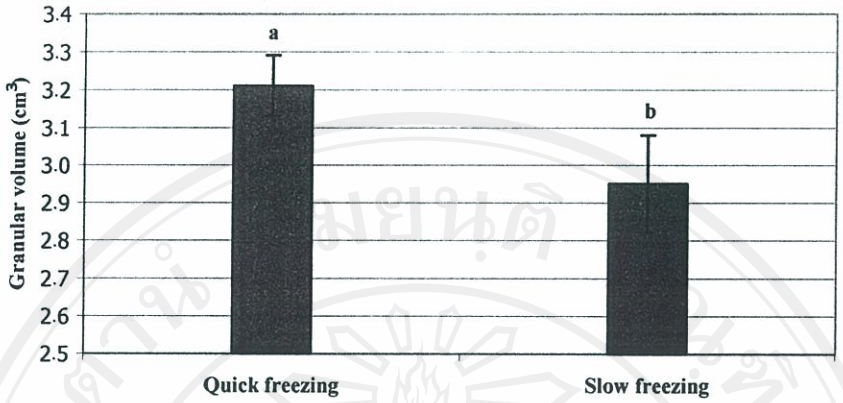

Figure 13 Effect of freezing methods on the granular volume of FDTB.

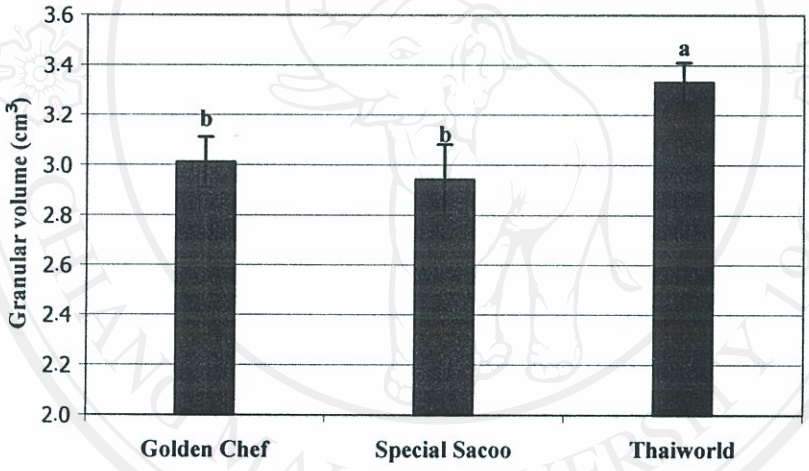

Figure 14 Effect of commercial brands on the granular volume of FDTB.

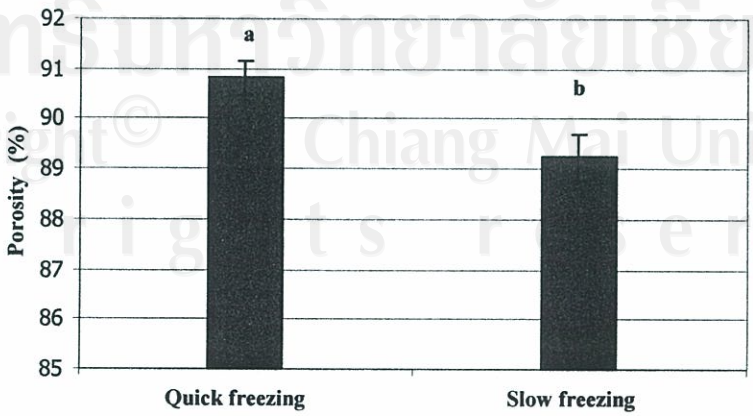

Figure 15 Effect of freezing methods on the porosity of FDTB.

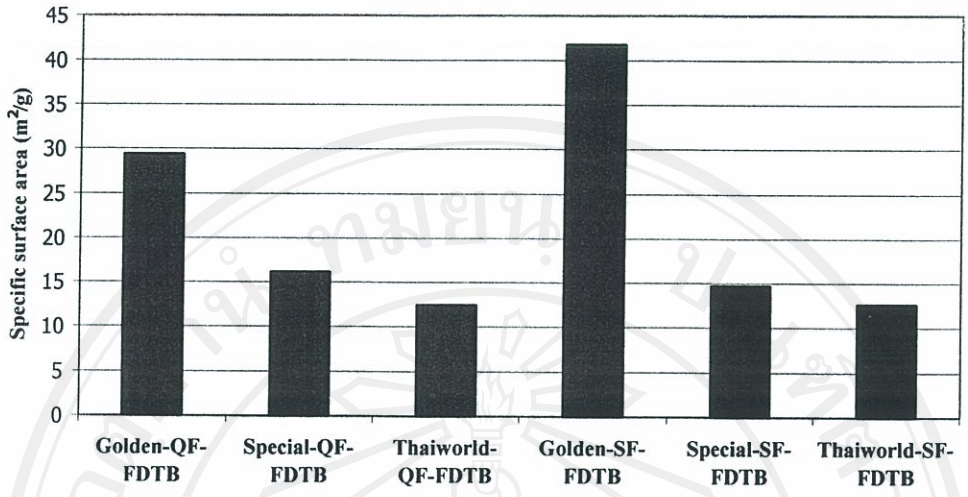

Figure 16 Effects of commercial brands and freezing methods on the specific surface area of FDTB.

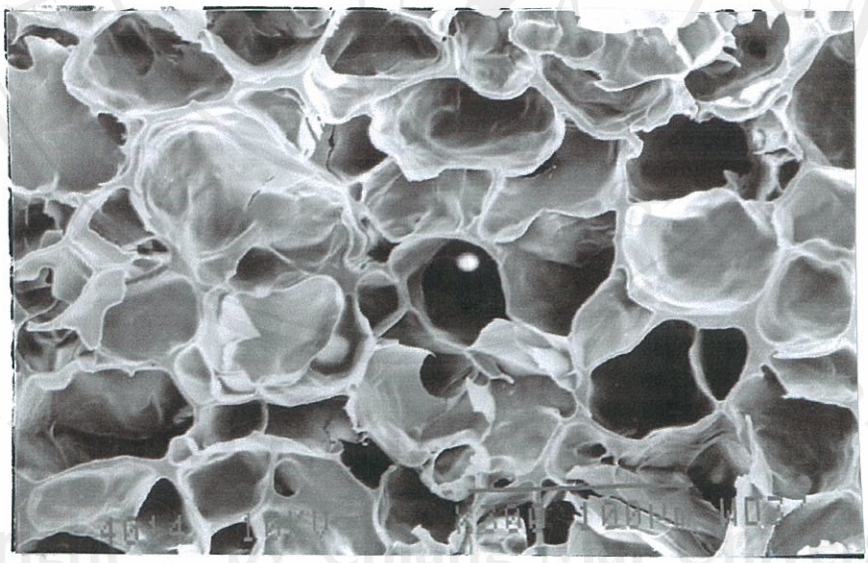

Figure 17 Scanning electron micrograph of cross-section of SF-FDTB at magnification x 300.

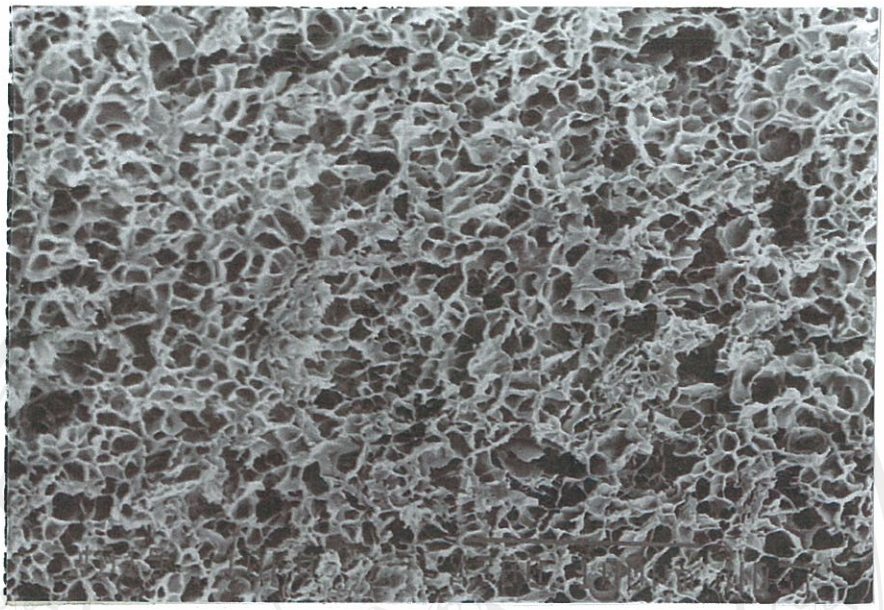

Figure 18 Scanning electron micrograph of cross-section of QF-FDTB at magnification x 300.

For preliminary understanding of the mechanisms involved in immobilization of bifidobacterial cells in QF- and SF-FDTB from Golden Chef®, Special Sacoo®, and Thaiworld®, adsorption capacity and adsorption behavior of QF- and SF-FDTB were determined. The statistical analysis showed the combined effect of three commercial brands and two freezing methods of FDTB on the adsorption capacity of 100-QF- and SF-FDTB is significant ($p \leq 0.05$) (Figure 19). The SF-FDTB had higher ($p \leq 0.05$) adsorption capacity than QF-FDTB for Special Sacoo® and Thaiworld®. The open porous structure of SF-FDTB may allows rapid and completed rehydration. While Golden Chef® had similarly ($P > 0.05$) result for both QF-FDTB and SF-FDTB. Three commercial brands and two freezing methods of FDTB were tested for adsorption behavior in PS at 4-5°C for 24 h. Similarly adsorption patterns of SF-FDTB and QF-FDTB from Golden Chef®, Special Sacoo®, and Thaiworld® were noticed (Figure 20). The adsorption rate of SF-FDTB was very fast in the first 50 min and the adsorption was maximum in 240 min. The maximum adsorption of QF-FDTB

was reached at 360 min. The mechanisms involved the water adsorption behaviors of QF- and SF-FDTB, which may be the hydrophilic property and water capillary attraction. Water may bind to the starch granule of QF- and SF-FDTB via hydrogen bonding which cause the beads to hold water inside the bead. The maximum adsorption of SF-FDTB reached faster than that of QF-FDTB. The reason may be due to the open surface characteristic and larger pore size of the SF-FDTB that allow water to penetrate more easily. The QF-FDTB had some parts of closed surface area and smaller pore size that may allow to water gradually penetrate into the beads. The maximum adsorption of QF- and SF-FDTB reached when the starch granules were fully hydrated. Once hydration occurred, hydrogen bonding between the amylose and amylopectin maintain the integrity of the granules and associate to form the matrix structure (Fennema, 1996). The matrix structure may present the beads to adsorb more water.

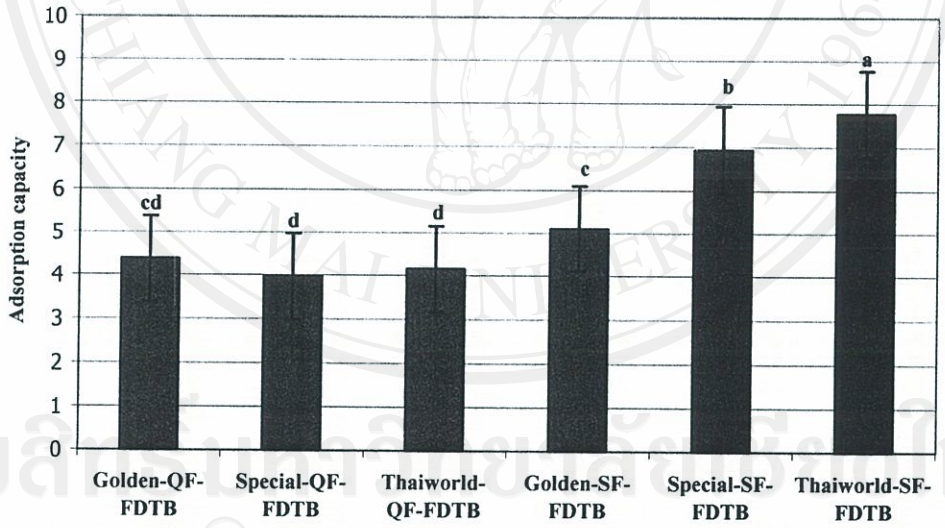

Figure 19 Effects of commercial brands and freezing methods on the adsorption capacity of 100-FDTB in PS, stored at 4-5°C.

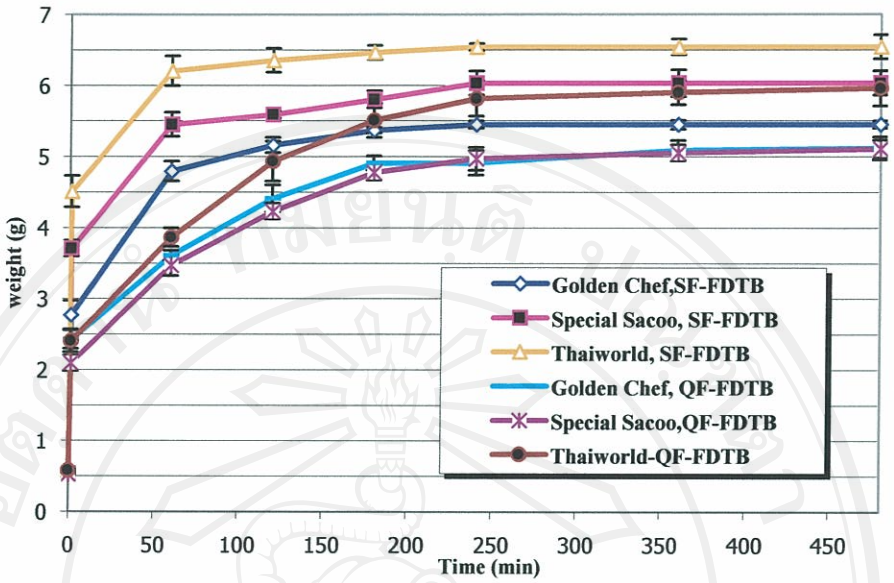

Figure 20 Effects of commercial brands and freezing methods on the adsorption behavior of 100-FDTB prior to store in PS at 4-5°C for 24 h.

After testing of adsorption behavior, gel strength of reformative QF- and SF-FDTB from Golden Chef®, Special Sacoo®, and Thaiworld® were determined. The gel strength of the three commercial brands produced by two freezing methods were significant difference ($p \leq 0.05$) (Figure 21). The firm texture was detected from the Golden Chef®FDTB processed by quick freezing and slow freezing. The gel strength of Golden Chef®QF-FDTB and SF-FDTB were 27.35 ± 1.33 and 22.18 ± 0.14 g, respectively. The lowest gel strength of 7.2 ± 0.39 g was detected from the Thaiworld®SF-FDTB. The large pore size of SF-FDTB created spongy texture, which had high adsorption property. The spongy texture caused decreasing of the gel strength of the rehydrated beads.

The QF- and SF-FDTB from three commercial brands were prepared that was used as supporting material for immobilization of bifidobacterial cells. However, QF- and SF-FDTB may have other beneficial effects for the other purposes that still need further study. The general advantages of QF- and SF-FDTB were: 1) people in many countries are familiar with TSB and use

gelatinized TSB as food and as a source of carbohydrate; 2) QF- and SF-FDTB have the benefits of being nontoxic to the cells being immobilized, and it is an accepted food ingredient; and 3) reformative QF- and SF-FDTB have soft and smooth texture which are easy to consume. Accordingly, TSB could be the ideal carrier for cells immobilization.

In conclusion, Quick- and slow-freezing methods contributed to the distinguished pore size, porosity, and surface characteristic of the QF- and SF-FDTB from all three commercial brands. The puffiness of the outer surface area, expanded dimension, and large pore size (ca 57.04 μm, diameter) were detected in SF-FDTB. In contrast, QF-FDTB showed the smooth outer surface area and very small pore size (ca 11.18 μm, diameter). Because of the combined effects of three commercial brands and two freezing methods were observed on the adsorption capacity of 100-FDTB. Six treatments of three commercial brands and two freezing methods of FDTB were selected for further study.

Figure 21 Effects of commercial brands and freezing methods on gel strength of the reformative FDTB prior to store in PS at 4-5°C for 16-18 h.

4.2 Effects of immobilization time and freezing methods of Special Sacoo® FDTB on the viability of immobilized *B. infantis*

To determine the optimum time of immobilization, the effect of time on the viability of *B. infantis* immobilized in Special Sacoo®QF- and SF-FDTB was studied. The samples were collected in 6, 18, and 24 h after put Special Sacoo®QF- or SF-FDTB into the cell suspension and stored at 4-5°C. The result showed in Figure 22. Each point in Figure 22 represents the mean from 3 replicates. Standard error bars are included.

The effect of immobilization time on the viable counts of *B. infantis* in Special Sacoo®QF- and SF-FDTB did not differ statistically ($P>0.05$). The adsorption mechanism of bifidobacterial cells into the porous beads could be explained by using the adsorption behavior of QF- and SF-FDTB in PS (Figures 19 and 20). When the 100-Special Sacoo®QF- and SF-FDTB were put into 5 mL bifidobacterial cell suspension, the Special Sacoo®SF-FDTB immediately adsorbed almost all the cell suspension into the porous beads within the first minute. While Special Sacoo®QF-FDTB gradually adsorbed the bifidobacterial cells into the porous beads. Figure 20 showed that the adsorptions of both SF-FDTB and QF- FDTB in PS at 4-5°C were maximum at 360 min. Then the immobilized beads were firstly collected at 360 min or 6 h. However, the quantity of the immobilized *B. infantis* after storage at 4-5°C for 18 h was detected more than those at 6 h for both Special Sacoo®QF- and SF-FDTB. Therefore, the immobilization time at 18 h was selected. Keeping the immobilized beads at 4-5°C could retard the growth and biochemical changes of bifidobacteria cells because bifidobacteria cannot grow at the temperature below 20°C (Ballongue, 1998). Gentle agitation of QF-FDTB in the cell suspension during 1-6 h of the immobilization, 5 s by vortex mixer, was necessary to prevent the sedimentation of bifidobacterial cell suspension.

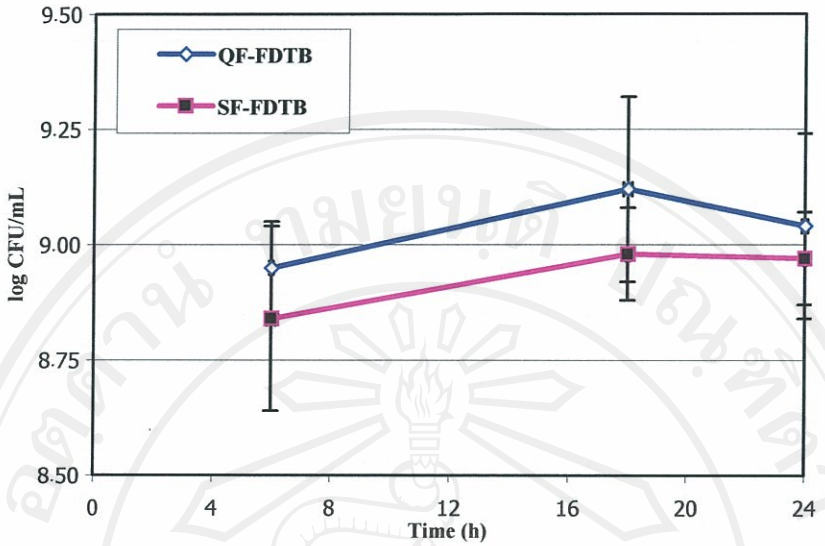

Figure 22 Effects of immobilized time and freezing methods on the viability of *B. infantis*. Cell suspension of *B. infantis* was adsorbed to Special Sacoo®QF- and SF-FDTB prior to store at 4-5°C for 24 h.

4.3 Effects of commercial brands and freezing methods of FDTB on the immobilization of *Bifidobacterium* spp.

Three commercial brands and two freezing methods of FDTB were studied in combination to investigate the viable count of bifidobacterial cells and the efficiency of immobilization. Three strains of bifidobacteria: *B. logum, B. bifidum*, and *B. infantis*, were used. The initial free cells in 5-mL PS for *B. longum, B. bifidum*, and *B. infantis* were 9.10, 9.15, and 9.46 log CFU/mL, respectively. After immobilization, the viable counts of *B. logum, B. bifidum*, and *B. infantis* were determined and showed the results in Figure 23. The percentage of immobilization was calculated and showed the results in Figures 24-25 and Table 3, respectively. Each bar in the Figures 23-25 represents the mean from 5 replicates. Standard error bars are included.

After immobilization, the viable counts of all strains showed similar results (P>0.05) (Figure 23). However, the average of QF-FDTB and SF-FDTB in 1 mL were 13 and 9 beads, respectively for all three commercial brands.

Then after calculation of the percentage of immobilization, the statistical analysis showed the effect of freezing method on the immobilization efficiency is significant ($p \leq 0.05$) (Figure 25). The SF-FDTB had higher ($p \leq 0.05$) percentage of immobilization than QF-FDTB for each tested bifidobacteria. Immobilized QF-FDTB had less cells ($p \leq 0.05$) than those of SF-FDTB for all tested strains (Figure 25). One possible reason is the swelling of the granular structure of QF- FDTB, which may occur during the adsorption of cell suspension. Figure 9 presented some part of closed outer surface area of QF-FDTB. The pore size of QF-FDTB may be decreased by the swollen granular structure especially at the surface area of the rehydrated beads. Decreasing of pore size could not allow the bifidobacterial cells to pass through the beads easily. In contrast, the open porous structure of SF-FDTB with the larger pore size would allow rapid adsorption.

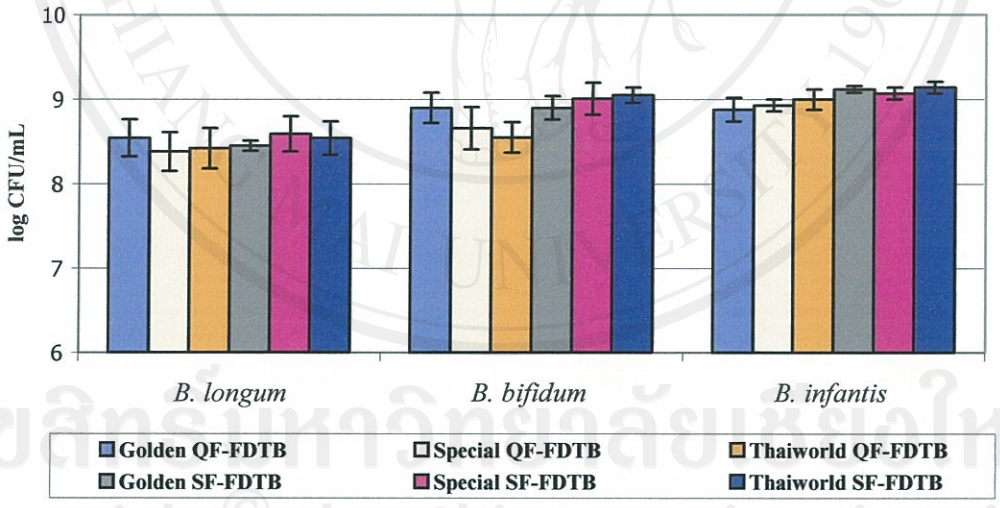

Figure 23 Effects of commercial brands and freezing methods of FDTB on the viability of immobilized *B. longum, B. bifidum,* and *B. infantis*, stored at 4-5°C for 16-18 h.

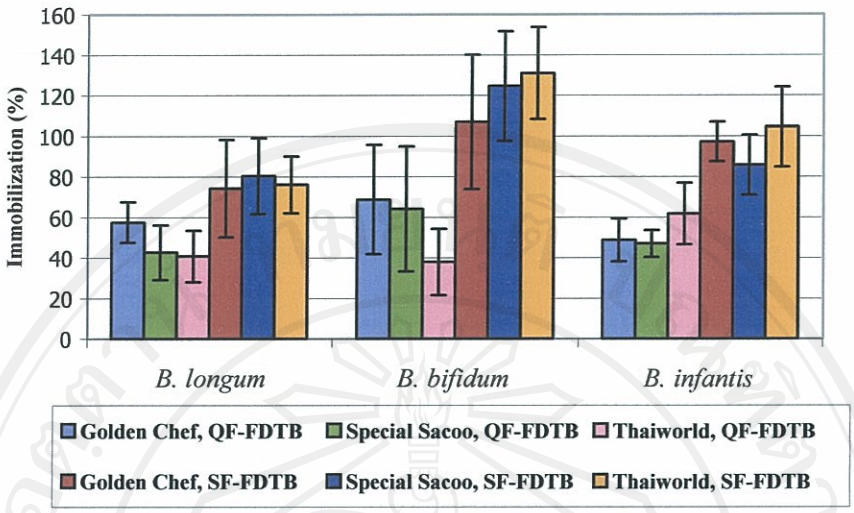

Figure 24 Effects of commercial brands and freezing methods of FDTB on the percentage of immobilization of *B. longum*, *B. bifidum*, and *B. infantis*, stored at 4-5°C for 16-18 h.

Table 3 Analysis of variance of effects of commercial brands and freezing methods of FDTB on the percentage of immobilization of *B. longum*, *B. bifidum*, and *B. infantis*, stored at 4-5°C for 16-18 h.

Source of variance	df	*B. longum*		*B. bifidum*		*B. infantis*	
		MS	p	MS	p	MS	p
Freezing method(A)	1	6679	0.03*	30682	0.01*	14210	0.00*
Brand(B)	2	137	0.90	254	0.93	713	0.46
AB	2	328	0.78	1899	0.60	66	0.94
Error	12	1307		3576		901	

* significant ($p \leq 0.05$)

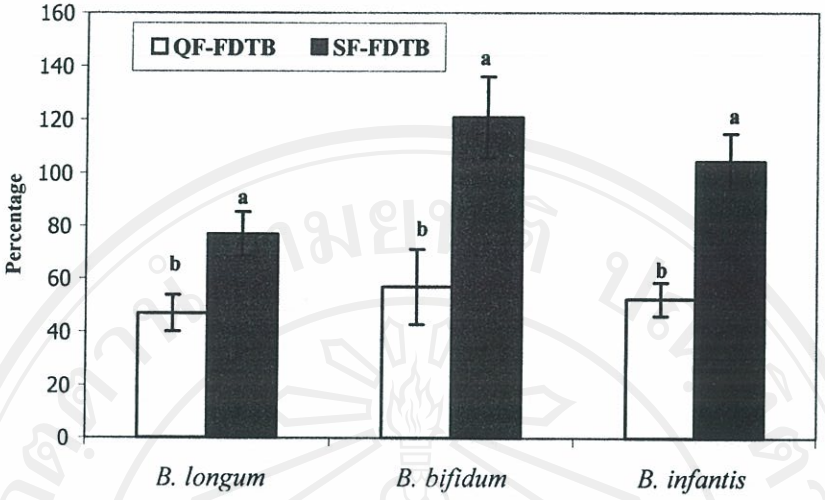

Figure 25 Effect of freezing methods of FDTB on the percentage of immobilization of *B. longum*, *B. bifidum*, and *B. infantis*, stored at 4-5°C for 16-18 h.

In this experiment, the percentage of immobilization of *B. longum*, *B. bifidum*, and *B. infantis* in QF- and SF-FDTB for all three commercial brands were determined. SF-FDTB showed the higher ($p \leq 0.05$) percentage of immobilization of bifidobacterial cells than QF-FDTB for all tested bifidobacteria (Figure 25). Because three commercial brands were not affected the percentage of immobilization of QF- and SF-FDTB. Whatever brand in three tested commercial brands could be used for further study. However, Special Sacoo®QF-FDTB and SF-FDTB were selected for further studying in order to decrease the inherent variation of samples. Special Sacoo® has the cheapest price when compared to Golden Chef® and Thaiworld®. In this experiment, the concentration of bifidobacterial cell suspension for each *Bifidobacterium* was fixed. The maximum capacity of QF- and SF-FDTB to hold the bifidobacterial cells was reported in experiment 4.4.

4.4 Effects of freezing methods of FDTB and bifidobacterial cell concentrations on the viability of immobilized *Bifidobacterium* spp. stored at 4-5°C for 16-18 h

The cell-load capacities of Special Sacoo®QF-FDTB and SF-FDTB were compared using *B. longum*, *B. bifidum*, and *B. infantis,* at various concentrations of 2x, 4x, 6x, 8x, and 10x, respectively and showed the results in Figures 26-30 and Table 4, respectively. Each point in Figures 26-28 and each bar in Figures 29-30 represent the mean from 3 replicates. Results with different letters are significantly different ($p \leq 0.05$ by Duncan's multiple range test). Standard error bars are included.

The results showed that the cell-load capacity of Special Sacoo®SF-FDTB was higher ($p \leq 0.05$) than that of Special Sacoo®QF-FDTB for *B. longum* and *B. infantis*. Whereas the cell-load capacity of Special Sacoo®QF-FDTB and Special Sacoo®SF-FDTB showed similar ($P > 0.05$) results for *B. bifidum* (Figure 29). The statistical analysis showed significant ($p \leq 0.05$) effect of cell concentrations on the viable counts of *B. infantis* in Special Sacoo®QF- and SF-FDTB (Figure 30). The lowest value ($p \leq 0.05$) was found at 2x cell concentration. The different results of cell-load capacity of Special Sacoo®QF- and SF-FDTB for *B. longum* and *B. infantis* when compared to *B. bifidum* may be affected by the adhesion ability of the bifidobacterial cells to granular starch. Crittenden *et al.* (2001) reported that not all bifidobacteria could adhere to the granular starch, and the adhesion does not appear to be a requirement for starch utilization by all strains in bifidobacterial genus. Cell surface proteins of bifidobacteria that specifically bind to alpha 1,4-linked glucose saccharides are involved in adhesion of the bacteria to the starch rather than nonspecific hydrophobic or electrostatic interactions. Specific starch-binding proteins have been observed in another intestinal bacterium, *Bacteroides thetaiotaomicron*, that produces a number of noncatalytic outer membrane proteins involved in starch adhesion. The nature of the cell surface proteins and their role in starch catabolism by

bifidobacteria remain to be explored (Crittenden *et al.*, 2001). Shimamura *et al.* (1992) reported that bifidobacterial cells could utilize carbohydrates when exposed to oxygen, even though they could not increase numbers by cell division due to oxygen toxicity. Adhesion to the starch was considered a possible mechanism for increasing bifidobacterial survival (Wang *et al.*, 1999).

The cell-load capacity showed the advantage of QF- and SF-FDTB to hold the high quantity of bifidobacterial cells within the beads. Immobilization of high concentration of bifidobacterial cells in QF- or SF-FDTB in this experiment required about 16-18 h, and yet used very simple technique for immobilization. Using QF- or SF-FDTB for immobilizing high quantity of bifidobacterila cells may be the advantage alternative compared to using alginate beads or using the method of Maitrot *et al.* (1997). The problems of using alginate beads for cell immobilization were reported (Hannoun and Stephanopoulos, 1986; King *et al.* ,1989; Seifert and Phillips, 1997; Ki-Yong and Tae-Ryeon, 2000). Maitrot *et al.* (1997) immobilizied *B. longum* in k-carrageenan / locust bean gum gel beads and cultured in a medium containing MRS broth and whey permeate. Viable population in the beads was 10.67 log CFU/g after fermentations. Increasing bifidobacterial cells inside the beads by using 3-5 successive batch fermentation requires additional time. The maximum concentration of cells loaded in Special Sacoo® SF-FDTB were $2.6x10^9$, $3.9x10^9$, and $8.4x10^8$ bifidobacterial cells per bead for *B. longum*, *B. bifidum*, and *B. infantis*, respectively. Gardiner *et al.* (2002) reported that the minimum level of viable bacteria is approximately 10^6 bifidobacteria per mL of product at the time of consumption in order to provide functional properties of probiotic. The suggested therapeutic dose is 10^8–10^9 viable bifidobacterial cells per day. Then the maximum concentration of cells loaded of tested bifidobacteria in Special Sacoo®SF-FDTB per bead are suitable to carry the cultures and delivery to the human body.

In conclusion, Special Sacoo®SF-FDTB showed higher ($p \leq 0.05$) cell load capacity than Special Sacoo®QF-FDTB (Figure 29). Then Special Sacoo® SF-FDTB was selected for studying in the experiment 4.5.

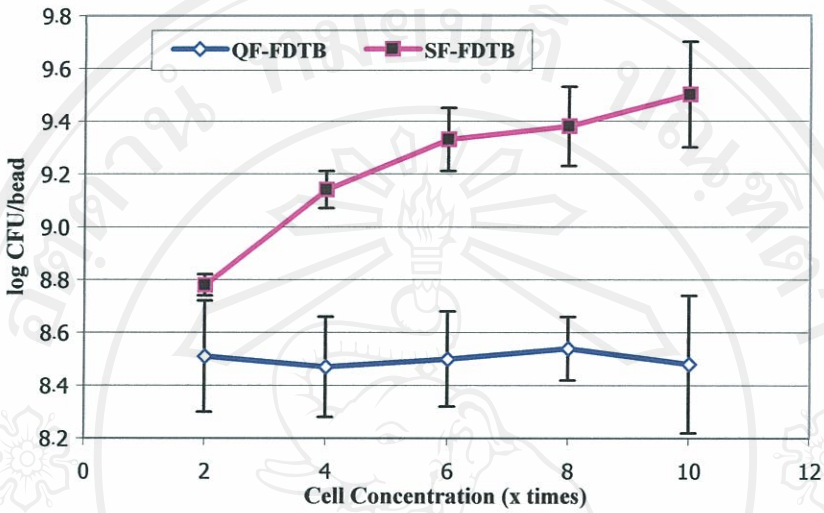

Figure 26 Effects of bifidobacterial cell concentrations and freezing methods of FDTB on the viability of immobilized *B. longum* that stored in the bifidobacterial cell suspension at 4-5°C for 16-18 h.

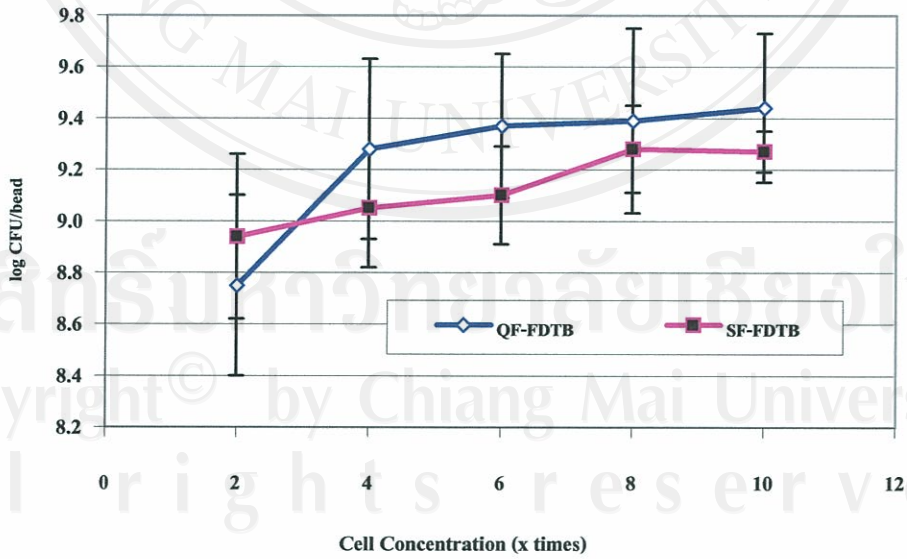

Figure 27 Effects of bifidobacterial cell concentrations and freezing methods of FDTB on the viability of immobilized *B. bifidum* stored at 4-5°C for 16-18 h.

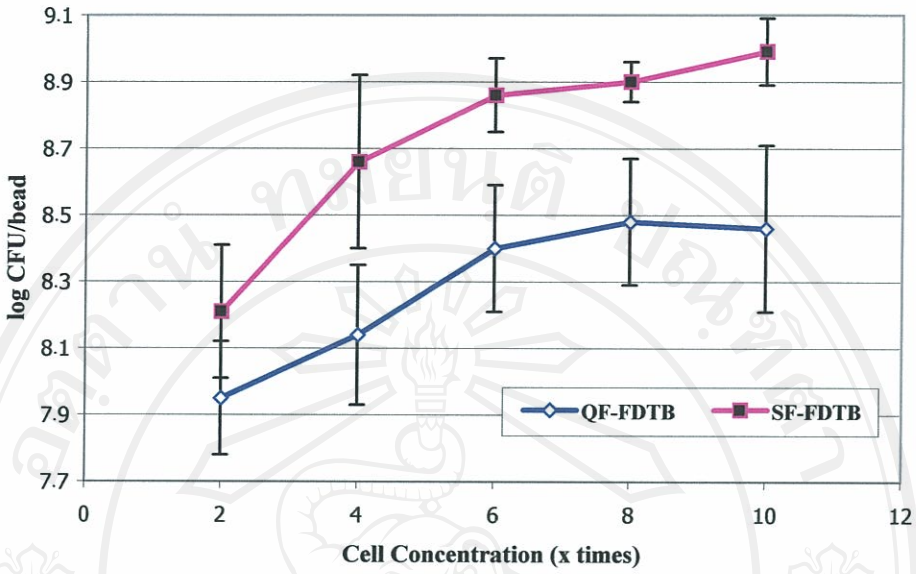

Figure 28 Effects of bifidobactrial cells concentration and freezing method of FDTB on the viability of immobilized *B. infantis* stored at 4-5°C for 16-18 h.

Table 4 Analysis of variance of effects of cell concentrations and freezing methods of FDTB on the viability of immobilized *B. longum*, *B. bifidum*, and *B. infantis* stored at 4-5°C for 16-18 h.

Source of variance	df	*B. longum*		*B. bifidum*		*B. infantis*	
		MS	p	MS	p	MS	p
Freezing method(A)	1	3.97	0.00*	0.10	0.51	1.48	0.00*
Cell concentration(B)	4	0.12	0.27	0.26	0.38	0.44	0.01*
AB	4	0.12	0.26	0.05	0.93	0.02	0.95
Error	20	0.08		0.23		0.10	

* significant ($p \leq 0.05$)

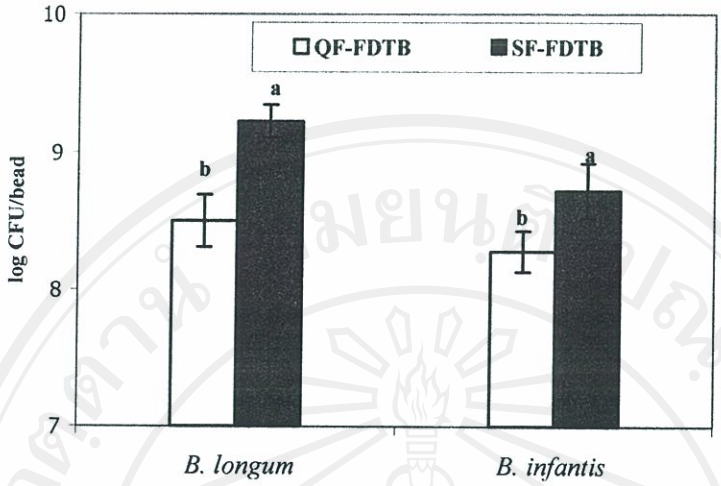

Figure 29 Effect of freezing methods of FDTB on the viability of immobilized *B. longum* and *B. infantis* stored at 4-5°C for 16-18 h.

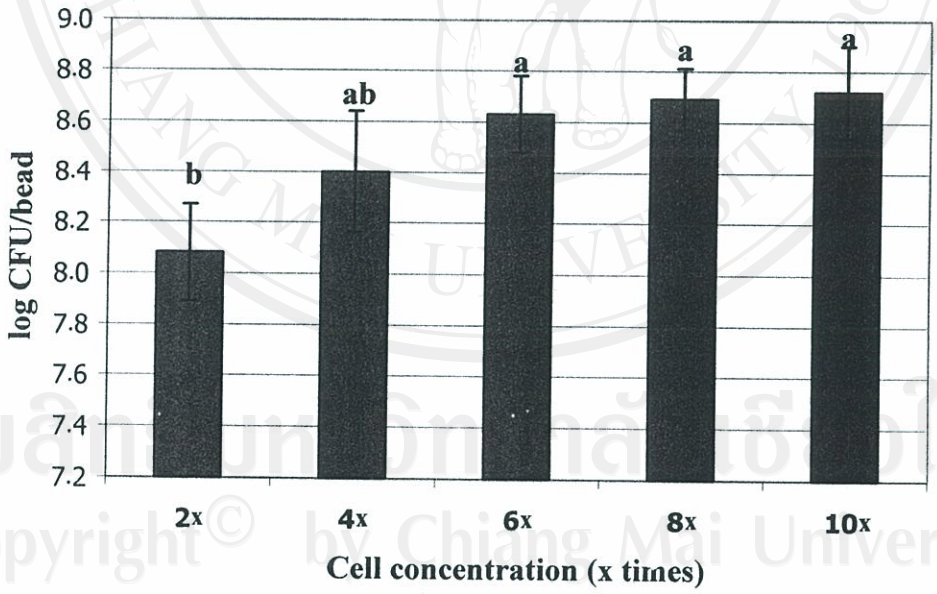

Figure 30 Effect of bifidobacterial cell concentrations on the viability of immobilized *B. infantis* in FDTB stored in the bifidobacterial cell suspensions at 4-5°C for 16-18 h.

4.5 Effect of freeze-drying on the viability of immobilized *Bifidobacterium* spp.

The viability of *B. longum*, *B. bifidum*, and *B. infantis* immobilized in fresh beads and dried beads were determined and showed the results in Figure 31. Each bar in Figure 31 represents the mean from 3 replicates. Results with different letters are significantly different ($p \leq 0.05$ by Duncan's multiple range test). Standard error bars are included.

The statistical analysis showed the effect of freeze drying on the viability of *B. longum*, *B. bifidum*, and *B. infantis* in Special Sacoo®SF-FDTB is significant ($p \leq 0.05$) when compared with the fresh beads. The viable counts of freeze dried-immobilized *B. longum*, *B. bifidum*, and *B. infantis* was lower ($p \leq 0.05$) than that of fresh beads for all tested bifidobacteria. The reduction of the viable counts of dried immobilized beads was ca 1 log-cycle when compared to the viable count of the fresh beads. The viable counts of freeze-dried-immobilized *B. longum*, *B. bifidum*, and *B. infantis* Special Sacoo®SF-FDTB were 9.13±0.13, 8.85±0.23, and 8.81±0.12 log CFU/mL, respectively.

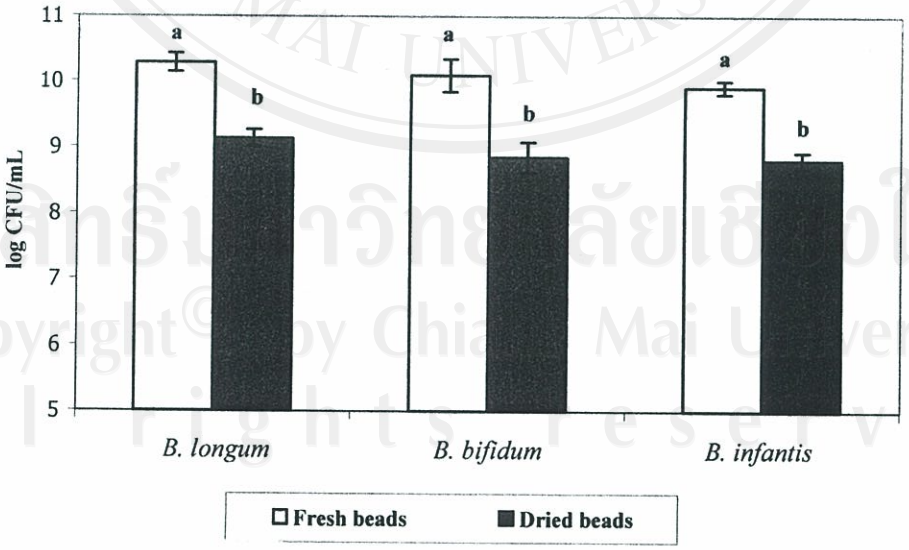

Figure 31 Effect of freeze-drying on the viability of immobilized *B. longum*, *B. bifidum*, and *B. infantis* in Special Sacoo®SF-FDTB.

4.5.1 Scanning electron micrograph of freeze-dried-immobilized *Bifidobacterium* spp.

The cross-section of immobilized dried beads were examined by using SEM and showed the results in Figures 32-35, respectively.

The scanning electron micrograph in Figure 32 and 33 showed the rod shape of dried *B. longum* and immobilized *B. longum* in Special Sacoo®SF-FDTB. Figure 34, 35 showed 1 mm and 2 mm dept cross-section of immobilized *B. bifidum* in Special Sacoo®SF-FDTB, respectively. Figures 35 indicated that bifodobacterial cells could reach the middle of the beads. Most of bifidobacterial cells adhered at the outer area, approximately 1 mm from the surface. The microstructure of immobilized bifidobacteria was also reported by Hansen *et al.* (2002). Hansen *et al.* (2002) determined the structure of fresh alginate microencapsulated *Bifidobacterium lactis* by using cold-stage scanning electron microscopy (cryo-SEM). Cryo-SEM showed the rod shape of *B. lactis* and void spaces around the bacteria in alginate microspheres.

The open porous structure of Special Sacoo®SF-FDTB may allow oxygen to enter the beads. Using of edible bilayer films to coat the dried-immobilized beads could prevent cell from oxygen exposure, and protect cells from acidic condition of yogurt or severe condition of simulated gastrointestinal system in the next experiment. The protective coating could also prevent leaking of the immobilized cells during storage and use. The leakage may occur due to a sponge like texture with the big pore size and weak binding force between the cells and the Special Sacoo®SF-FDTB.

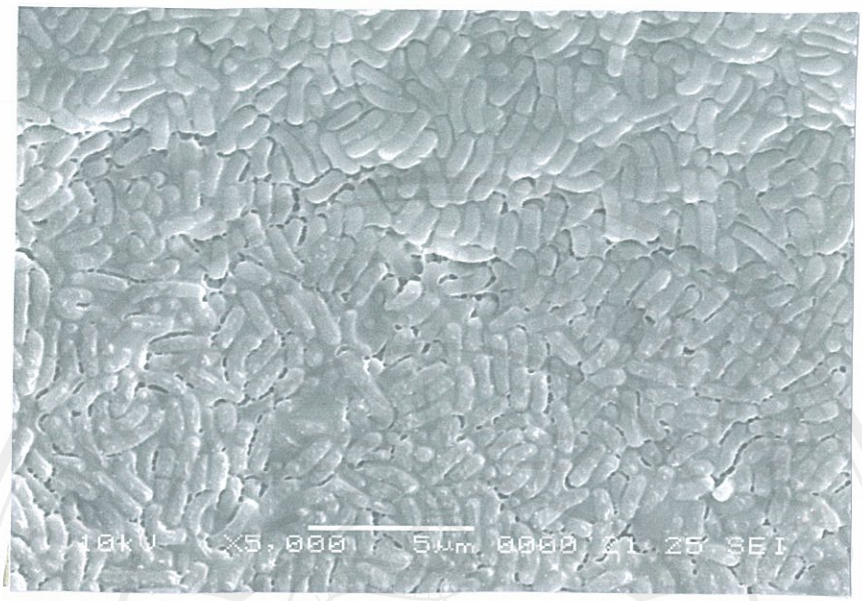

Figure 32 Scanning electron micrograph of dried *B. longum* at magnification x 5000.

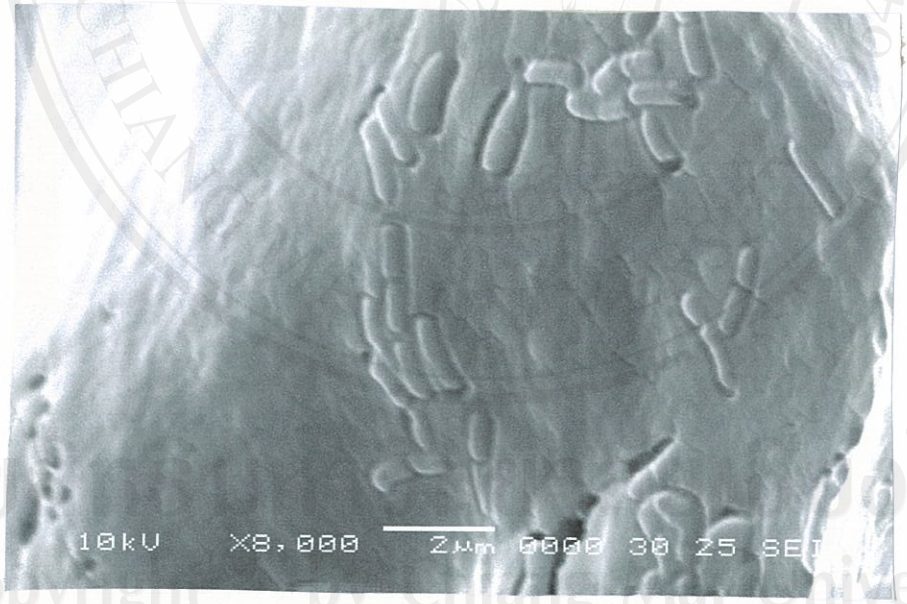

Figure 33 Scanning electron micrograph of cross-section of immobilized *B. longum* in Special Sacoo®SF-FDTB at magnification x 8,000.

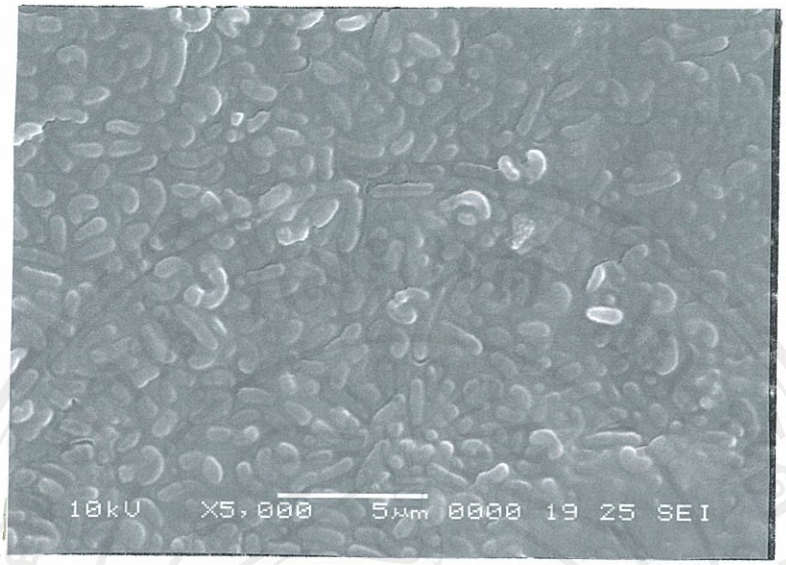

Figure 34 Scanning electron micrograph of cross-section of immobilized *B. bifidum* in Special Sacoo®SF-FDTB (1 mm depth) at magnification x 5,000.

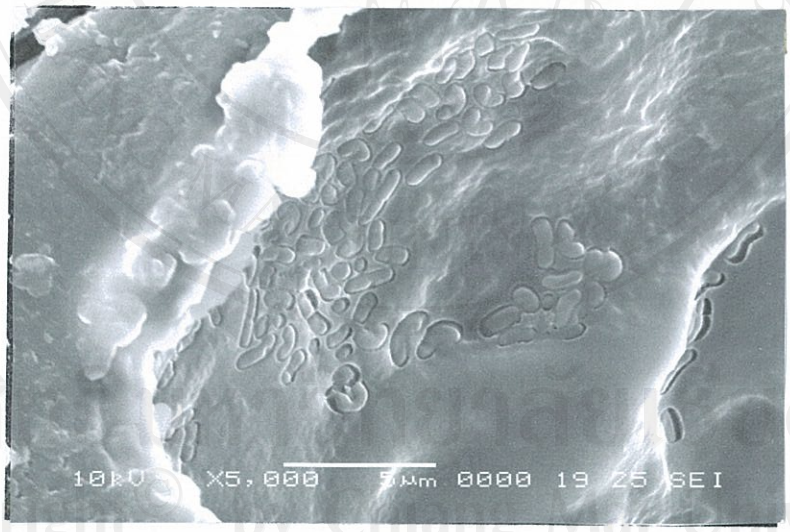

Figure 35 Scanning electron micrograph of cross-section of immobilized *B. bifidum* in Special Sacoo®SF-FDTB (2 mm depth) at magnification x 5,000.

4.6 Effect of coating materials on the viability of dried-immobilized *Bifidobacterium* spp.

After drying, the dried-immobilized-beads were then coated with bilayer films from fats (palmitic acid, PANODAN®, or beeswax) and sodium caseinate. The purpose of coating dried-immobilized beads with bilayer film from fat and sodium caseinate was to protect bifidobacterial cells from oxygen exposure, severe condition of simulated gastrointestinal fluid and acid condition of yogurt. Sequence of coating, coating with sodium caseinate followed by fat coating or in contrast, coating with fat followed by sodium caseinate, were studied. All fat coating materials, palmitic acid, PANODAN®, and beeswax, were tested. Coating dried-immobilized beads with sodium caseinate film solution as the first layer caused the shrinkage of the porous beads. The diameter reduced from 4 mm to 2 mm after drying. The bead had hard texture. After following by coating, the bilayer-coated beads were tested for the adhesion of coatings to the beads. The beads were immersed in PS for 1-2 h. The results showed that the fat coating was separated from the beads for all tested fat coating materials. In contrast, coating dried bead with fat first could maintain the structure of the porous beads. However, during coating of fat, the melted fat was adsorbed into the beads. The sodium caseinate and fat (palmitic acid, PANODAN® or beeswax) bilayer film-coated beads had a white color and glossy surface area. And the bilyer films did not separate from the beads after testing in PS for 1-2 h. Coating with fat firstly and followed by sodium caseinate was selected. The weight of coating materials were determined and showed the results in Figure 36. The effects of coating materials on the viability of dried-immobilized *B. longum*, *B. bifidum*, and *B. infantis* in Special Sacoo®SF-FDTB were determined and showed the results in Figure 37. Each bar in the Figure 36 represents the mean from 9 replicates. Each bar in the Figure 37 represents the mean from 3 replicates. Results with different letters are

significantly different ($p<0.05$ by Duncan's multiple range test). Standard error bars are included

Coating weights of palmitic acid, PANODAN® and beeswax showed similar results ($P>0.05$) (Figure 36). The average coating weight of palmitic acid, PANODAN® and beeswax per bead was 0.02 g. Coating with fats (palmitic acid, PANODAN®, or beeswax) and sodium caseinate had no effect ($P>0.05$) on the viable counts of *B. longum* and *B. infantis* but had effect ($p<0.05$) on the viable count of *B. bifidum*. Coating with PANODAN® provided the lowest ($p<0.05$) viable count of *B. bifidum*. Coating with fat (palmitic acid, PANODAN® or beeswax) was performed at 50-55°C within 10 s. This condition could stabilize the viable count of *B. longum* and *B. infantis*. Decreasing of *B. bifidum* after coating with PANODAN® could not discuss because lacking of the support information about the properties of PANODAN®.

In conclusion, coating with edible fat (palmitic acid, PANODAN® or beeswax) and sodium caseinate had no effect ($P>0.05$) on the viable count of *B. longum*, and *B. infantis* but had effect ($p<0.05$) on the viable count of *B. bifidum* in Special Sacoo®SF-FDTB. PANODAN® coating decreased the viable count of *B. bifidum*.

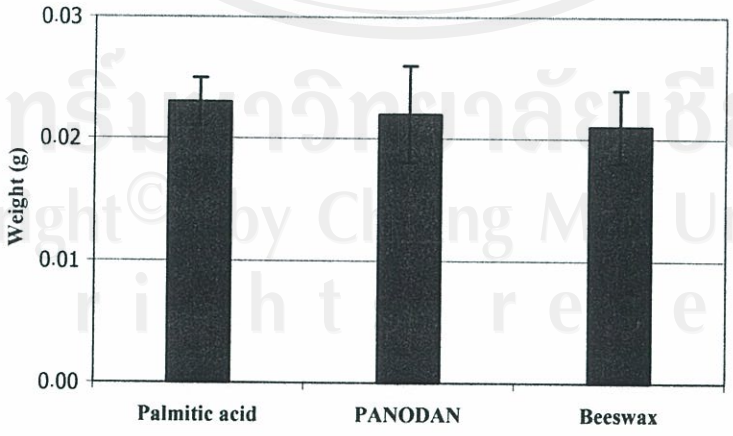

Figure 36 Weight of coating materials on the coated-immobilized beads.

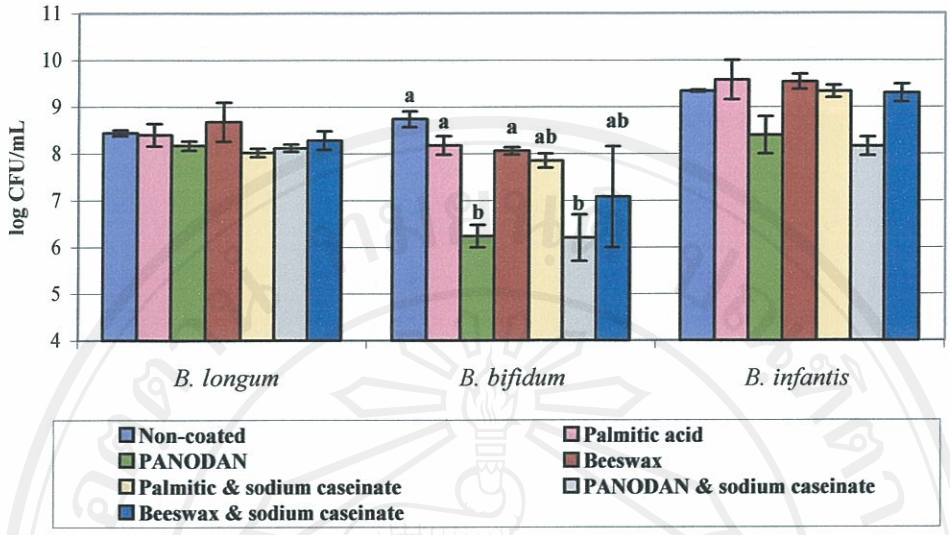

Figure 37 Effect of coating materials on the viability of immobilized *B. longum*, *B. bifidum*, and *B. infantis* in Special Sacoo®SF-FDTB.

4.6.1 Scanning electron micrograph of coated-immobilized beads

The outer surface area of coated immobilized beads were examined by using SEM and showed the results in Figures 38-40, respectively.

The scanning electron micrograph in Figure 38 showed the crystallized structure of palmitic acid coated-immobilized bead. Whereas Figure 39 showed the smooth-continuous layer of beeswax. The moisture and gas barrier properties of lipid coatings are dependent on the packing of the lipid crystals (Baldwin *et al.*, 1997). The crystallization of beeswax is in a dense-orthorhombic arrangement and has more effective as a gas and vapor barrier than PANODAN® and palmitic acid (Gontard *et al.*, 1994; Hernandez, 1994). However, the barrier properties of beeswax may decreased due to film's defect (Figure 39). The defect of beeswax coating may occur during using of forceps to carry the coated beads. Figure 40 showed the closed-smooth area of sodium caseinate film cover the surface area of beeswax coated-immobilized beads. McHugh and Krochta (1994) reported that caseinate films provided transparent and

flexible films. However, the crack of sodium caseinate film was observed in this study. The crack could decrease the barrier properties of this film.

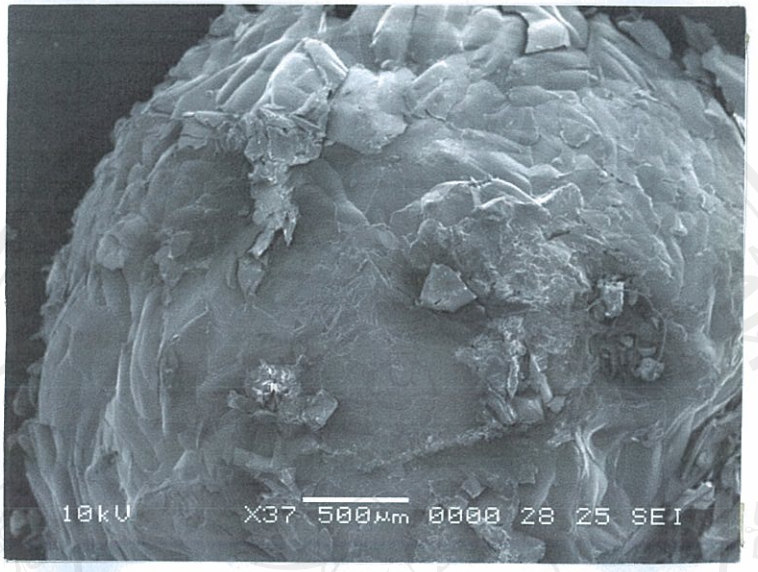

Figure 38 Scanning electron micrograph of outer surface area of palmitic acid coated immobilized bead at magnification x 37.

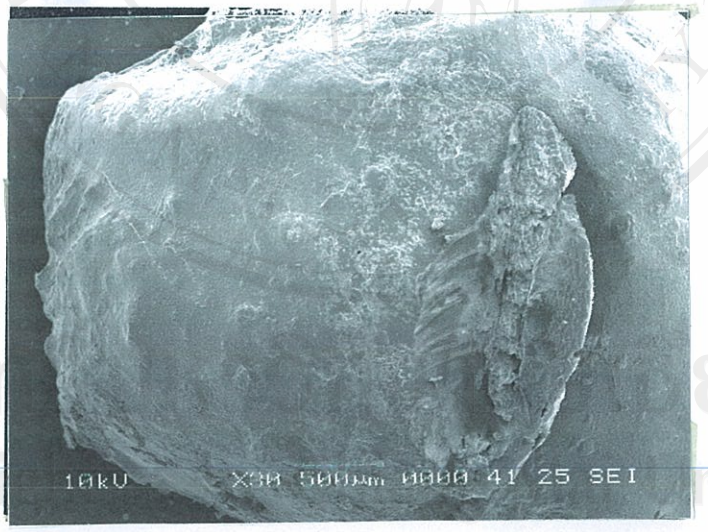

Figure 39 Scanning electron micrograph of outer surface area of beeswax coated-immobilized bead at magnification x 30.

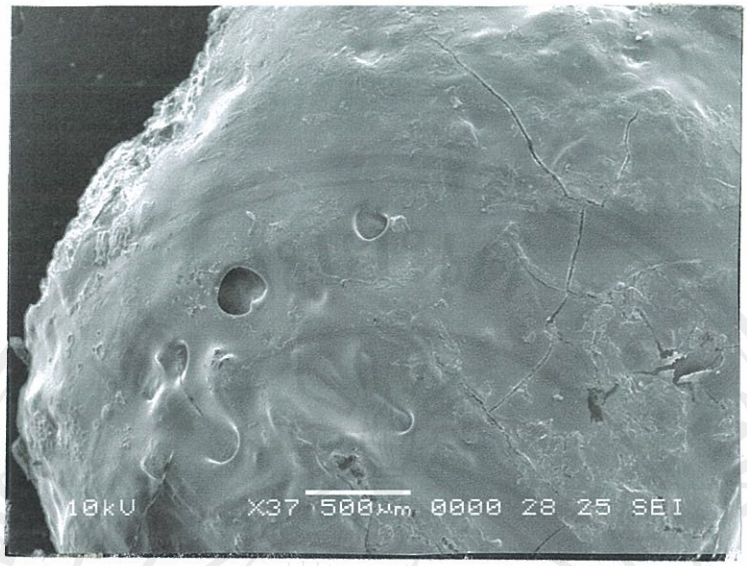

Figure 40 Scanning electron micrograph of outer surface area of sodium caseinate coating of immobilized bead after coating with beeswax at magnification x 37.

4.7 Survival of free cells, non-coated, and coated-dried-immobilized *Bifidobacterium* spp. in simulated gastrointestinal fluids without enzyme at 37°C for 310 min

The survival of free cells, non-coated, and coated-dried-immobilized *B. longum*, *B. bifidum*, and *B. infantis* in simulated gastrointestinal fluids without enzyme were determined and showed the results in Figure 41. Each bar in Figure 41 represents the mean from 3 replicates. Results with different letters are significantly different ($p \leq 0.05$ by Duncan's multiple range test). Standard error bars are included.

Significant protection ($p \leq 0.05$) of *B. longum*, *B. bifidum*, and *B. infantis* by immobilization with and without the coating of bilayer films were observed (Figure 41). The viable count of free cells of *B. longum* was lower ($p \leq 0.05$) than that of non-coated and coated-dried-immobilized *B. longum* ca 2.0 log-cycle. The viable count of free cells and coated-immobilized beads of *B. bifidum* with PANODAN® and sodium caseinate were lower ($p \leq 0.05$) than that of non-coated, coated immobilized-beads with palmitic acid and sodium caseinate

and with beeswax and sodium caseinate ca 1.0-2.0 log-cycle. The viable count of free cells of *B. infantis* was lower ($p \leq 0.05$) than non-coated and coated-dried-immobilized *B. infantis* for all material coatings ca 1.5-2.0 log-cycle. Sodium caseinate and beeswax-coated-immobilized *B. infantis* had the higher ($p \leq 0.05$) viable count than non-coated-immobilized *B. infantis* ca 0.5 log CFU/mL. The survival in simulated gastrointestinal fluid without enzyme of non-coated and coated-immobilized bifidobacterial cells were higher ($p \leq 0.05$) that of free cells for all tested bifidobacteria, except the coated immobilized *B. bifidum* with PANODAN® and sodium caseinate. The viable counts of *B. longum*, *B. bifidum*, and *B. infantis* were ca 3.0 ± 0.2, 2.8 ± 0.4, and 3.5 ± 0.2 log CFU/mL, respectively after exposed to simulated gastrointestinal fluids. Whereas the viable counts of immobilized *B. longum*, *B. bifidum*, and *B. infantis* were 5.0 ± 0.1, 3.0-5.1 ± 0.5, and 5.1 ± 0.1 log CFU/mL, respectively after exposed to simulated gastrointestinal fluids. The protection mechanism of immobilized beads and bilayer coating with bacterial cells could explain by the basic principle of immobilization and coating techniques that reported by Pszczola (2003). The gel matrix of Special Sacoo®SF-FDTB could act as the protective layer of the cells, to prevent or retard the acidic solution of simulated gastric fluid to contact the cells. The coating materials are designed to stabilize and protect immobilized bifidobacterial cells. Coating immobilized cells with the edible bilayer films could act as the additional protective layer and may let the coated immobilized cells expose to the simulated gastric fluid shorter than free cells. However, the function of sodium caseinate layer may be decreased in simulated gastric fluid (pH 1.2). Sodium caseinate was separated from the beads due to loss of adhesive property. Sodium caseinate could not dissolve at pH less than 5.5 (McHugh and Krochta, 1994).

The results from this experiment showed the advantage of immobilization in Special Sacoo®SF-FDTB with or without the combination of bilayer coating when compared to the experiments of Sun and Griffiths (2000) and Hansen

et al. (2002). Sun and Griffiths (2000) reported that immobilized *B. infantis* ATCC 15697 in 3 mm gellan-xanthan bead could not survive in simulated gastric fluids when stored at pH 1.5 for 15 min. Hansen *et al.* (2002) reported that the viable counts of free cells and immobilized *B. infantis* Bb-02, *B. longum* Bb-46, and *B. bifidum* Bb-11 in alginate microencapsulated beads (20 and 70 μm) were < 1.0±0.0, <1.0±0.0, and 3.2±0.6 log CFU/mL, respectively after exposed to simulated gastric fluid (USP without pepsin, pH 2.0 at 37°C for 120 min). High survival (ca 8.9 log CFU/mL) of 0.5-1 mm microencapsulated *B. infantis* in Ca-alginate after exposed to milk based simulated gastric medium (12% non-fat skim milk, 2% glucose, 1% yeast extract, and 0.05% cysteine) at pH 2.0 without pepsin was reported by Sultana *et al.* (2000).

There are number of simulated gastric formula and conditions available in the literature. There is no standard formula and method for testing the survival of bifidobacteria in simulated gastrointestinal system. The USP simulated gastrointestinal fluids were selected in this experiment following the standard method of pharmacy. Using USP simulated gastrointestinal fluids could determine the effects of pH and transit time simulated human gastrointestinal tract on the survival of *B. longum*, *B. bifidum*, and *B. infantis*. The effect of enzyme in the simulated gastrointestinal fluids was not determined in this experiment because pepsin is stable at pH 2.5. Whereas the USP simulated gastric fluid was carried out at pH 1.2, which was very severe condition for pepsin. Recent works in food and pharmaceutical science that used simulated gastric fluid without pepsin, for example, Hansen *et al.* (2002); Sultana *et al.* (2000); Sun and Griffiths (2000); Ki-Yong and Tae-Ryeon (2000); Sriamornsak *et al.* (1997); Rao *et al.* (1989). However, the effect of enzyme in simulated gastrointestinal fluid on the survival of probiotic culture should be studied in the future. The standard formula and method of simulated gastrointestinal system for testing the survival of probiotic should be set in order to standardize the results from many research.

In conclusion, the results from this experiment indicated that the immobilization technology with or without the combination of edible bilayer coating could protect tested bifidobacteria effectively from the severe condition of simulated gastrointestinal fluids without enzyme. In order to confirm the survival of coated-immobilized bifidobacteria, the future experiment should be carried out in the *in vivo* experiment.

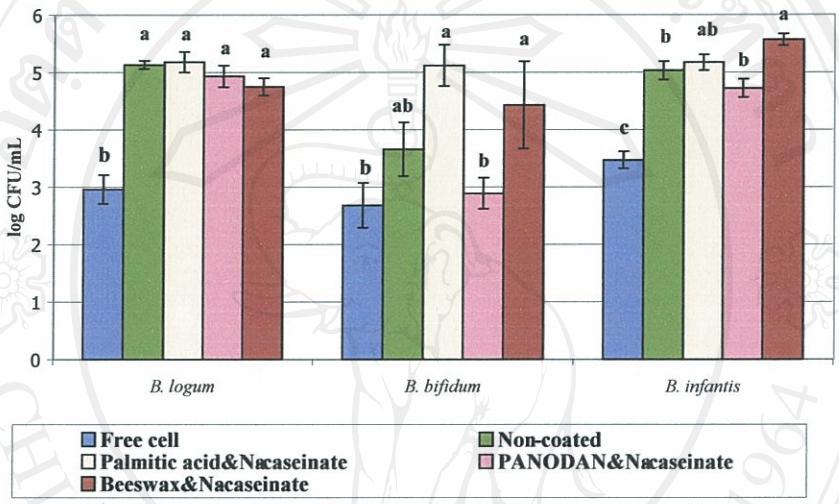

Figure 41 The survival of free cells, non-coated, and coated-dried-immobilized *B. longum*, *B. bifidum*, and *B. infantis* in simulated gastrointestinal fluids without enzyme at 37°C for 310 min.

4.7.1 Scanning electron micrograph of coated-immobilized beads

The cross-section of coated immobilized beads after storage in simulated gastrointestinal fluids without enzyme for 310 min were examined by using SEM and showed the results in Figures 42-43.

The scanning electron micrograph in Figure 42 showed the porous structure of Special Sacoo®SF-FDTB with various pore sizes. The variation of pore sizes may be affected by two freezing-steps of the samples. The attachment of *B. infantis* on the surface area inside the porous beads after testing with simulated gastrointestinal fluids at 37°C for 310 min, showed in Figure 43.

The rod shape of *B. infantis* appeared under SEM at a magnification of x 5,000. The less population of *B. infantis* was observed. This may be due to some of bifidobacteria die after exposure to the severe condition of simulated gastric fluid (pH 1.2) and may lose their adhesive property to the surface area of Special Sacoo®SF-FDTB.

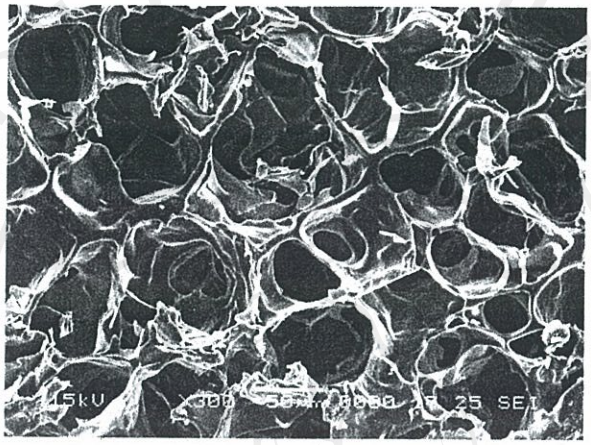

Figure 42 Scanning electron micrograph of the cross-section of coated immobilized bifidobacteria in Special Sacoo®SF-FDTB after storage in simulated gastrointestinal fluids without enzyme for 310 min, at magnification x 300.

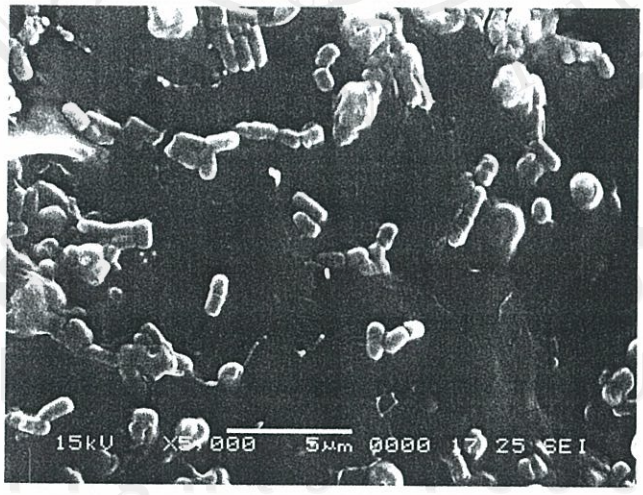

Figure 43 Scanning electron micrograph of the cross-section of sodium caseinate caseinate and beeswax-coated-immobilized *B. infantis* in Special Sacoo®SF-FDTB after storage in simulated gastrointestinal fluids without enzyme for 310 min, at magnification x 5,000.

4.8 Effects of coating materials and storage time on the survival of non coated and coated-immobilized *Bifidobacterium* spp. in Special Sacoo® SF-FDTB during storage in pasteurized yogurt at 4-5°C for 4 wk and in simulated gastrointestinal fluid at 37°C for 310 min

The survival of non-coated and coated-immobilized *B. longum*, *B. bifidum*, and *B. infantis* in Special Sacoo®SF-FDTB during storage in pasteurized yogurt at 4-5°C for 4 wk were determined and showed the results in Figures 44-48 and Table 5, respectively. The pH of pasteurized yogurt was monitored throughout the period of storage time of 4 wk and showed the results in Figures 49-52 and Tables 6, respectively. Each point in the Figure 44 and each bar in Figures 45-52 represent the mean from 3 replicates. Results with different letters are significantly different ($p \leq 0.05$ by Duncan's multiple range test). Standard error bars are included.

The statistical analysis showed the effect of coating materials on the survival of immobilized *B. longum* in pasteurized yogurt, stored at 4-5°C for 4 wk, is significant ($p \leq 0.05$) (Figure 44). The statistical analysis showed the effect of storage time on the survival of immobilized *B. longum* in pasteurized yogurt, stored at 4-5°C for 4 wk, is significant ($p \leq 0.05$) (Figure 45). The viable count of immobilized *B. longum* was decreased ($p \leq 0.05$) ca 2 log-cycle after storage time. After storage for 4 wk, the viable count of immobilized *B. longum* was ca 6 log CFU/mL. Coating with PANODAN® and sodium caseinate provided the lowest ($p \leq 0.05$) viable count of immobilized *B. longum* (Figure 46). The effect of coating with PANODAN® and sodium caseinate on the viable counts of immobilized *B. longum* could not discuss because lack of the support information. The acidity and heat-holding capacity of PANODAN® should be investigated. The decreasing of viable counts of non-coated and coated-immobilized *B. longum* during storage may be affected by the low pH (pH 4.1-4.2) of pasteurized yogurt (Gilliland, 1979) and some amount of oxygen that may be in the packaging or could permeate to the packaging during

storage time (Ishibashi and Shimamura, 1993). There has no report about the survival of *B. longum* in pasteurized yogurt. However, the results from this experiment showed higher survival of bifidobacteria when compared the results with the report of Carr *et al.* (1999). Carr *et al.* (1999) studied the viability of bifidobacteria in bio-yogurt produced in North Carolina, USA. The results showed that 44 out of 58 or 75.9% commercial bio-yogurt contained viable cultures of bifidobacteria. The counts for bifidobacteria were varied among the tested samples ranging from 0 to 5.0 log CFU/mL.

The statistical analysis showed the combined effects of the coating materials and storage time on the viability of non-coated and coated immobilized *B. bifidum* and *B. infantis* in pasteurized yogurt, stored at 4-5°C for 4 wk are significant difference ($p \leq 0.05$) (Figures 47 and 48). The viable counts of non-coated and coated-immobilized *B. bifidum* decreased ($p \leq 0.05$) ca 1 log-cycle while that of *B. infantis* decreased ($p \leq 0.05$) ca 2 log-cycle after stored in pasteurized yogurt at 4-5°C for 4 wk. The lower ($p \leq 0.05$) viable counts of immobilized *B. bifidum* and *B. infantis* were observed in the coated samples with PANODAN® during storage time.

The viabilities of non-coated and coated-immobilized bifidobacteria stored in pasteurized yogurt at 4-5°C for all treatments and all tested bifidobacteria were gradually declined within 4 wk (Figures 44-45 and 47-48). After storage for 4 wk, the viable counts of bifidobacteria were ca 6-7 log CFU/mL. The results agree with the work of Sun and Griffiths (2000) who reported that the viable counts of immobilized *B. infantis* ATCC 15697 in pasteurized yogurt after stored at 4°C for 4 wk was ca 6.0 log CFU/mL. The decreasing of viable counts of non-coated and coated-immobilized *B. bifidum* and *B. infantis* during storage may be affected by the low pH (pH 4.1-4.2) of pasteurized yogurt (Gilliland, 1979) and some amount of oxygen that may be in the packaging or could permeate to the packaging during storage time (Ishibashi and Shimamura, 1993). Sanders (1999) also discovered that *Bifidobacterium* is intolerant to high acidity

and generally has a limited capability to stabilize in fermented milk products. The weak cells of *B. longum, B. bifidum*, and *B. infantis* may die during storage in pasteurized yogurt.

The results of the free cells was not reported due to the interferent by the others anaerobic bacteria in the tested yogurt, CP-Meiji brand and the yogurt produced by the modified method of Sun and Griffith (2000). The interfered cultures may come from the facultative anaerobic spore forming bacteria of the genus *Bacillus* which could survive pasteurization. The similar interferant was reported by Noriega *et al.* (2003) and Janstova and Lukasova (2001). *Bacillus cereus* is a predominant aerobic microorganism influencing the shelf life of pasteurized milk. *Bacillus* spp. cause a serious problem in milk industry because of the heat resistance of *Bacillus* spores (Noriega *et al.* ,2003; Janstova and Lukasova, 2001). The next experiment was designed to eliminate the interfered anaerobic culture by using sterilization at 121°C for 15 min before adding bifidobacterial cells.

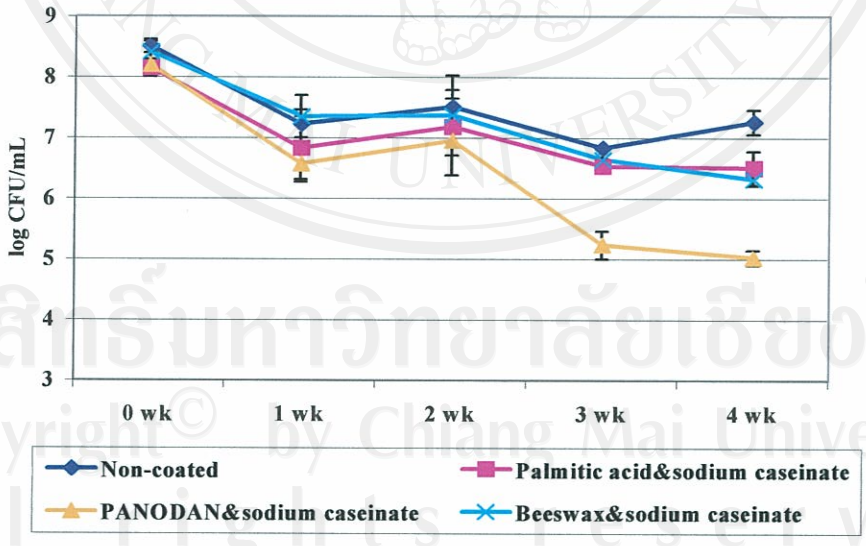

Figure 44 Effects of coating materials and storage time on the survival of non coated and coated-immobilized *B. longum* in Special Sacoo®SF-FDTB during storage in pasteurized yogurt at 4-5°C for 4 wk.

Table 5 Analysis of variance of the effects of coating materials and storage time on the survival of non-coated and coated-immobilized *Bifidobacteria* spp. in Special Sacoo®SF-FDTB during storage in pasteurized yogurt at 4-5°C for 4 wk.

Source of variance	df	*B. longum*		*B. bifidum*		*B. infantis*	
		MS	p	MS	p	MS	p
Storage time (A)	4	8.20	0.00*	6.43	0.00*	8.62	0.00*
Coating materials (B)	3	3.20	0.00*	8.74	0.00*	7.02	0.00*
AB	12	0.41	0.18	0.76	0.01*	0.99	0.01*
Error	40	0.28		0.27		0.39	

* significant ($p \leq 0.05$)

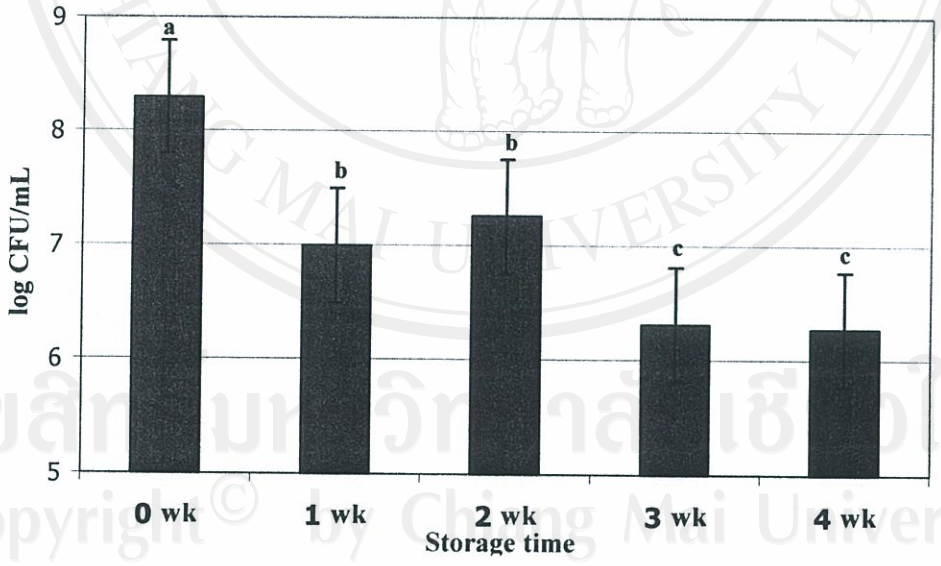

Figure 45 Effect of storage time on the survival of immobilized *B. longum* in Special Sacoo®SF-FDTB during storage in pasteurized yogurt at 4-5°C.

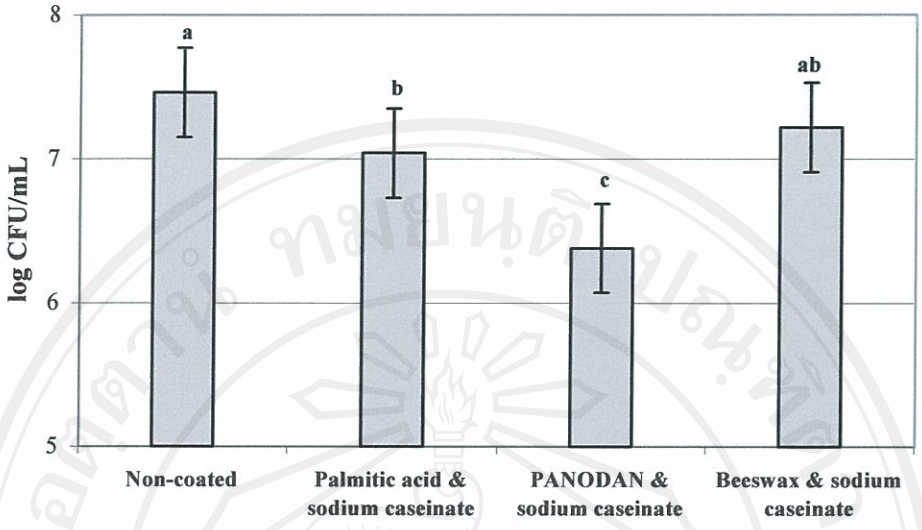

Figure 46 Effect of coating materials on the survival of immobilized *B. longum* in Special Sacoo®SF-FDTB during storage in pasteurized yogurt at 4-5°C for 4 wk.

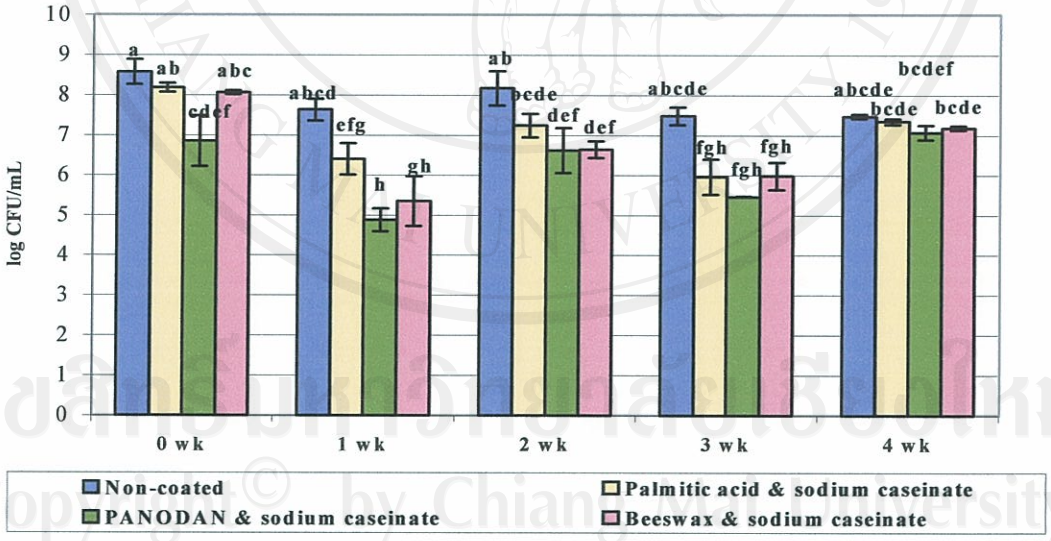

Figure 47 Effects of coating materials and storage time on the survival of non coated and coated-immobilized *B. bifidum* in Special Sacoo®SF-FDTB during storage in pasteurized yogurt at 4-5°C.

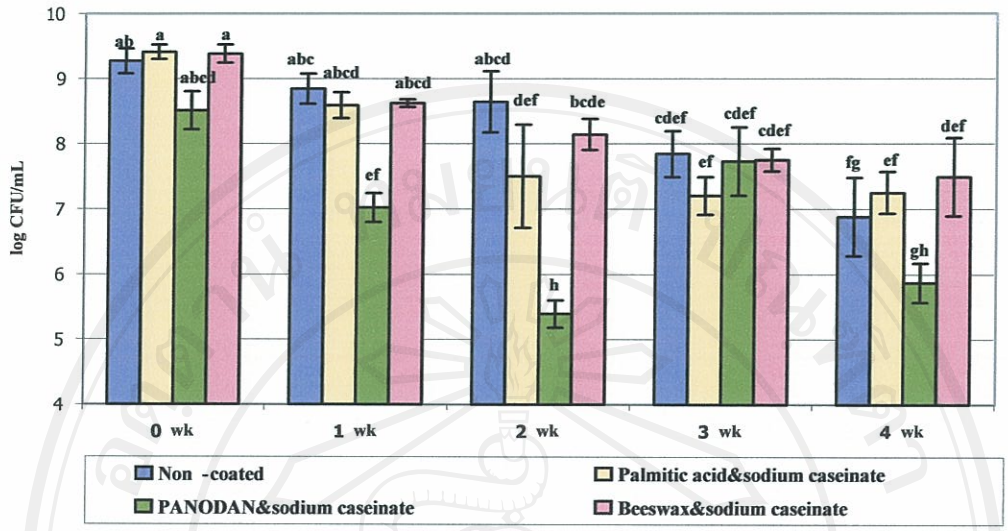

Figure 48 Effects of coating materials and storage time on the survival of non-coated and coated-immobilized *B. infantis* in Special Sacoo®SF-FDTB storage in pasteurized yogurt at 4-5°C.

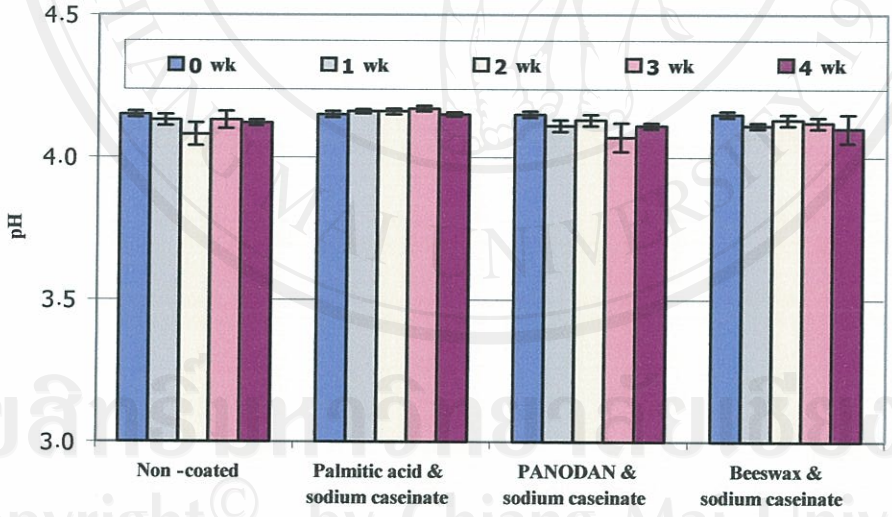

Figure 49 Effects of coating materials and storage time on pH of the pasteurized yogurt containing non-coated and coated-immobilized *B. longum* in Special Sacoo®SF-FDTB during storage in pasteurized yogurt at 4-5°C.

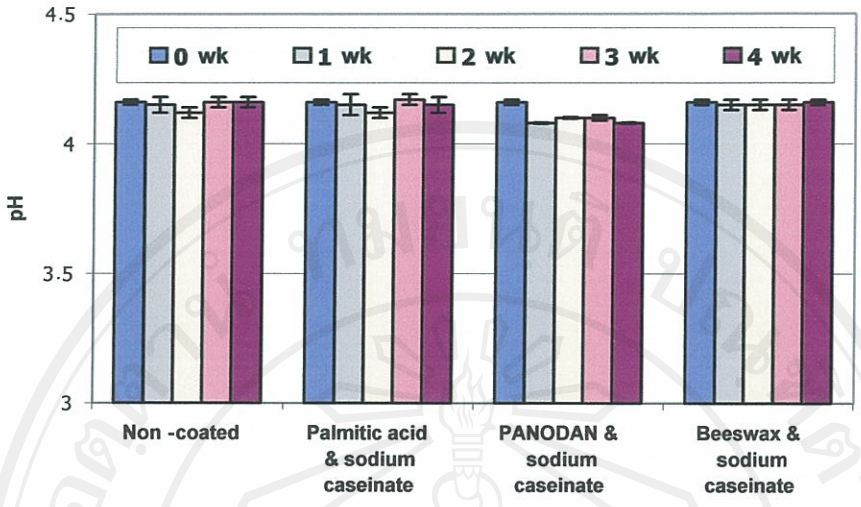

Figure 50 Effects of coating materials and storage time on pH of the pasteurized yogurt containing non-coated and coated-immobilized *B. bifidum* in Special Sacoo®SF-FDTB during storage in pasteurized yogurt at 4-5°C.

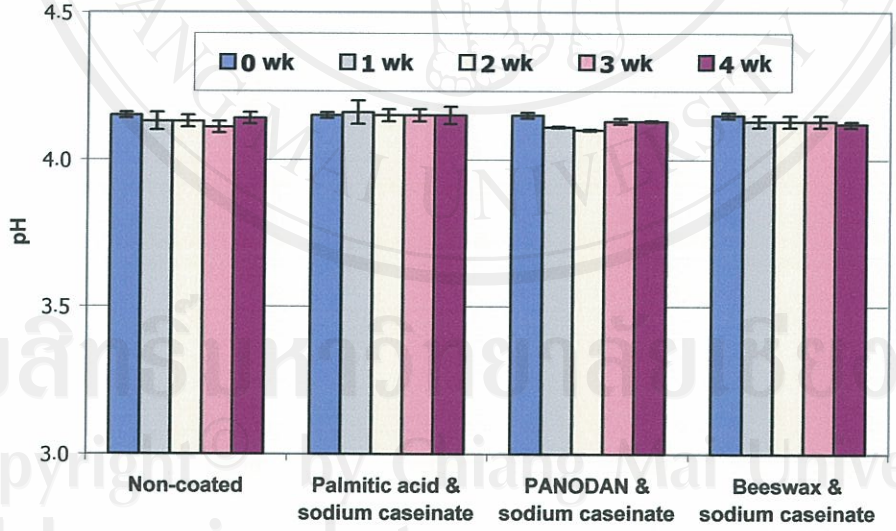

Figure 51 Effects of coating materials and storage time on pH of the pasteurized yogurt containing non-coated and coated-immobilized *B. infantis* in Special Sacoo®SF-FDTB during storage in pasteurized yogurt at 4-5°C.

Table 6 Analysis of variance of the effects of coating materials and storage time on the pH of the pasteurized yogurt containing non-coated and coated imobilized *Bifidobacteria* spp. in Special Sacoo®SF-FDTB during storage in pasteurized yogurt at 4-5°C.

Source of variance	df	*B. longum*		*B. bifidum*		*B. infantis*	
		MS	p	MS	p	MS	p
Storage time (A)	3	0.00	0.97	0.00	0.20	0.00	0.93
Coating materials (B)	3	0.01	0.00*	0.01	0.00*	0.02	0.00*
AB	9	0.00	0.10	0.00	0.56	0.00	0.13
Error	32	0.00		0.00		0.00	

* significant ($p \leq 0.05$)

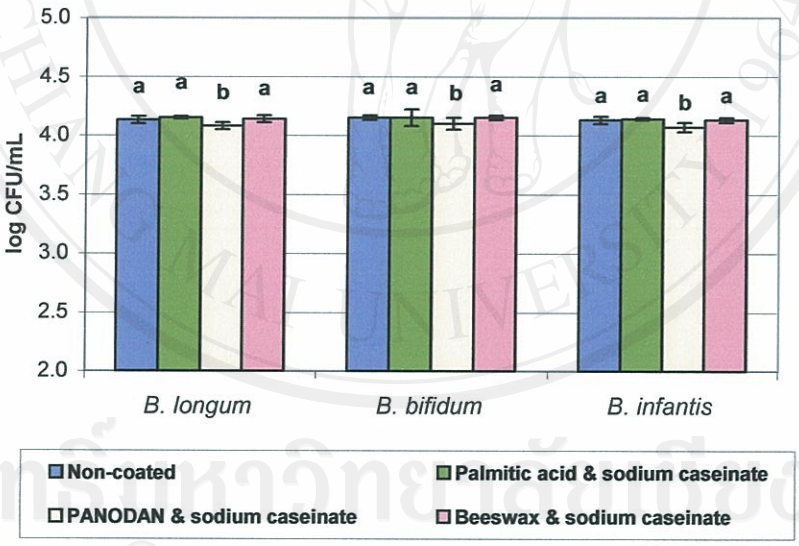

Figure 52 Effect of coating materials on pH of the pasteurized yogurt containing immobilized *B. longum*, *B. bifidum*, and *B. infantis* in Special Sacoo® SF-FDTB during storage in pasteurized yogurt at 4-5°C.

The statistical analysis showed only the effect of coating materials on changing of pH of the pasteurized yogurt containing immobilized *B. longum*, *B. bifidum* or *B. infantis* ($p \leq 0.05$). Coating with PANODAN® and sodium caseinate caused the lowest ($p \leq 0.05$) pH of pasteurized yogurt. Decreasing of pH may be affected by the acidity of PANODAN®. In this experiment, the post acidification by yogurt culture (*S. thermophilus* and *L. bulgaricus*) was eliminated. Pasteurized yogurt at 85-90°C for 30 min could kill *S. thermophilus* and *L. bulgaricus* (Sultana *et al.*, 2000; Siuta-Cruce and Goulet, 2001).

4.8.1 Scanning electron micrograph of non-coated immobilized bead after storage in pasteurized yogurt at 4-5°C for 4 wk

After storage in pasteurized yogurt for 4 wk, the samples of non-coated and coated-immobilized beads were collected and examined the microstructure of immobilized beads by SEM and showed the results in Figures 53-56.

The scanning electron micrograph showed the closed surface area of non-coated immobilized bead after storage in pasteurized yogurt for 4 wk (Figure 53). The outer surface of non-coated-immobilized bead differed from that of Special Sacoo®SF-FDTB in Figure 8. After immobilization with bifidobacterial cells and followed by freeze-drying, the puffiness of Special Sacoo®SF-FDTB was not observed. Immobilization of bifidobacterial cells may increase the compaction of intra-granular of Special Sacoo®SF-FDTB. Figure 54 showed a rough surface with some pores of sodium caseinate and palmitic acid coated immobilized bead. Figure 55 showed the non-smooth surface with large pore sizes of sodium caseinate and PANODAN®coated-immobilized bead. Figure 56 showed the non-smooth surface without pore of sodium caseinate and beeswax coated immobilized bead. The structure of bilayer films (Figures 53-56) changed during kept in the high moisture condition of yogurt when compared to the Figures 38-40.

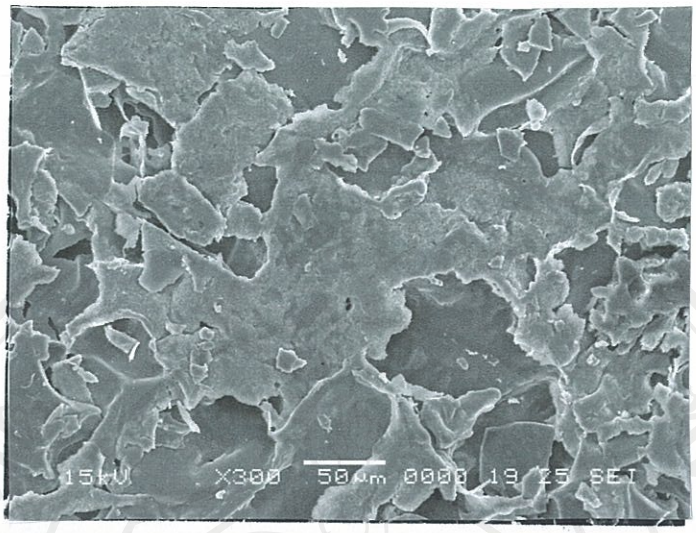

Figure 53 Scanning electron micrograph of the outer surface of non-coated immobilized bifidobacteria in Special Sacoo®SF-FDTB after storage in pasteurized yogurt at 4-5°C for 4 wk, at magnification x 300.

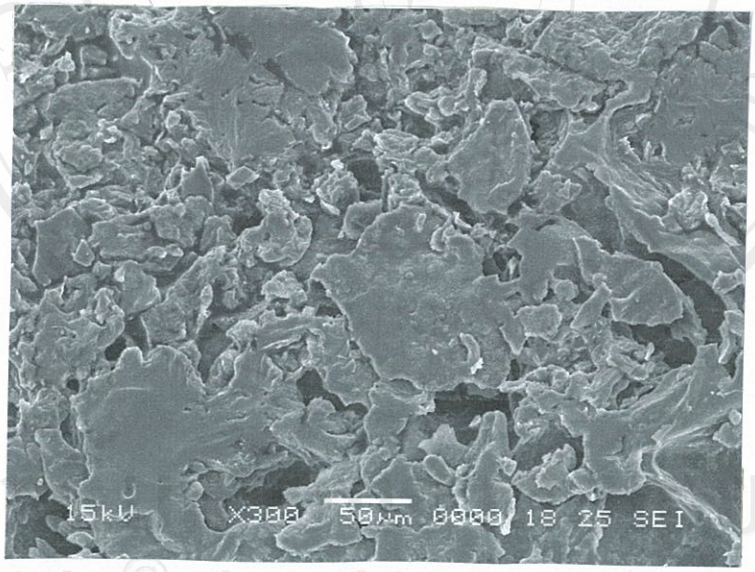

Figure 54 Scanning electron micrograph of the outer surface of double layers coating with sodium caseinate and palmitic acid immobilized bifidobacteria in Special Sacoo®SF-FDTB after storage in pasteurized yogurt at 4-5°C for 4 wk, at magnification x300.

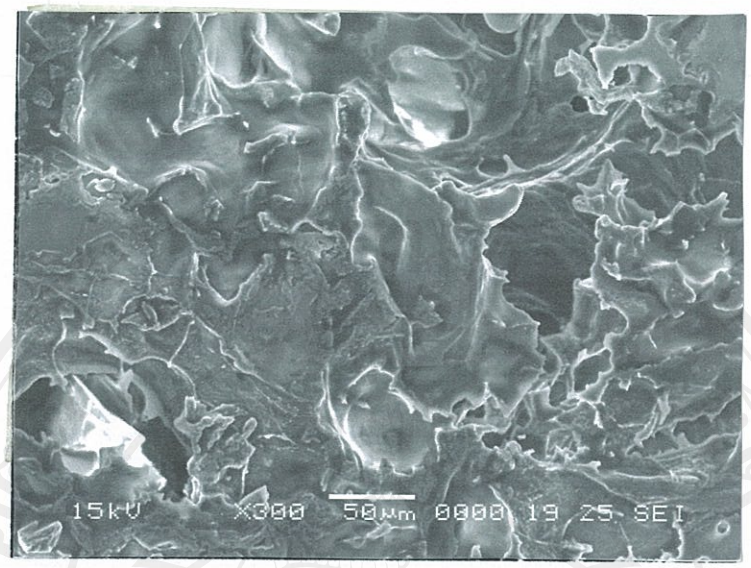

Figure 55 Scanning electron micrograph of the outer surface of sodium caseinate and PANODAN® coated-immobilized bifidobacteria in Special Sacoo®SF-FDTB after storag in pasteurized yogurt at 4-5°C for 4 wk, at magnification x 300.

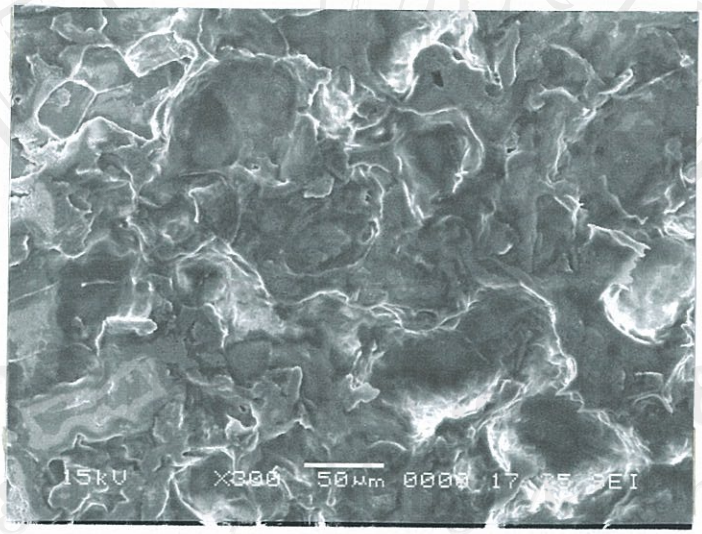

Figure 56 Scanning electron micrograph of the outer surface of sodium caseinate and beeswax coated-immobilized bifidobacteria in Special Sacoo®SF-FDTB after storage in pasteurized yogurt at 4-5°C for 4 wk, at magnification x 300.

4.8.2 Survival of non-coated and coated-immobilized *B. longum*, *B. bifidum*, and *B. infantis* after incubated in simulated gastrointestinal fluids without enzyme at 37°C for 310 min

After storage in pasteurized yogurt for 4 wk, the samples of non-coated and coated-immobilized beads were collected and tested for the viability in simulated gastrointestinal fluid without enzyme and showed the results in Figure 57. Each bar in Figure 57 represents the mean from 3 replicates. Standard error bars are included.

After storage in pasteurized yogurt at 4-5°C for 4 wk, the beads were collected and washed 3 times by PS to remove yogurt from the beads. Then the beads were incubated in simulated gastrointestinal fluids without enzyme at 37° C for 310 min. The survival of non-coated and coated-immobilized *B. longum*, *B. bifidum*, and *B. infantis* in Special Sacoo®SF-FDTB showed no significant difference (P>0.05). The results indicated that the edible bilayer films did not provide the extra protection to the immobilized cells.

In conclusion, non-coated and coated-immobilized *B. longum*, *B. bifidum*, and *B. infantis* in Special Sacoo®SF-FDTB could survive well after storage in pasteurized yogurt at 4-5°C for 4 wk. The viable counts of 2-5 log CFU/mL also found after incubation in simulated gastrointestinal fluids without enzyme at 37°C. Accordingly, the non coated-immobilized *bifidobacteria* spp. in Special Sacoo®SF-FDTB were selected to study in the experiment 4.9.

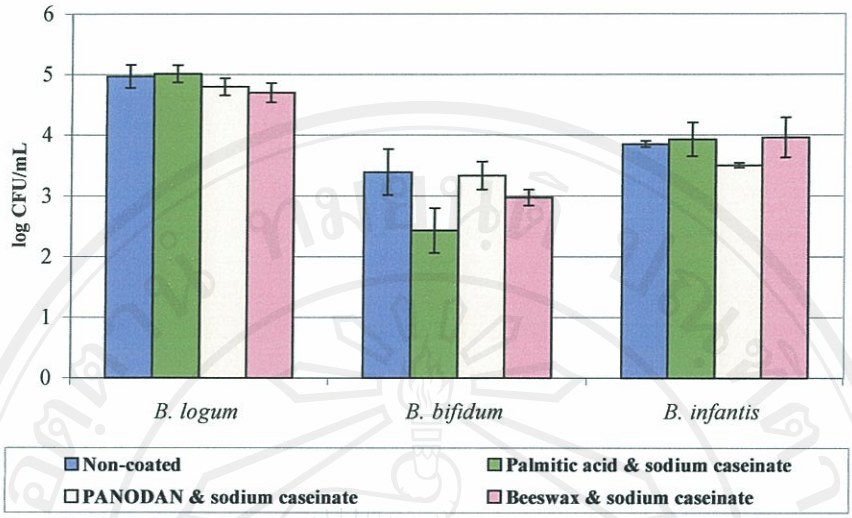

Figure 57 The survival of non-coated and coated-immobilized *B. longum*, *B. bifidum*, and *B. infantis* in Special Sacoo®SF-FDTB after storage in pasteurized yogurt at 4-5°C for 4 wk and incubated in simulated gastrointestinal fluids without enzyme at 37°C for 310 min.

4.9 Survival of free bifidobacterial cells and non-coated immobilized *Bifidobacterium* spp. in Special Sacoo®SF-FDTB during storage in sterilized yogurt at 4-5°C for 4 wk and in simulated gastrointestinal fluid without enzyme at 37°C for 310 min

The survival of free cells and non-coated-immobilized *B. longum*, *B. bifidum*, and *B. infantis* in Special Sacoo®SF-FDTB during storage in sterilized yogurt at 4-5°C for 4 wk and in simulated gastrointestinal fluids without enzyme at 37°C for 310 min, were determined and showed the results in Figures 58-60. Each bar in the Figures 58-60 represents the mean from 3 replicates. Results with different letters are significantly different ($p \leq 0.05$ by Duncan's multiple range test). Standard error bars are included.

The statistical analysis showed the significant effect ($p \leq 0.05$) of storage time on the survival of free cells of *B. longum* in Special Sacoo®SF-FDTB during storage in sterilized yogurt at 4-5°C for 4 wk (Figure 58). The viable

count of free cells were decreased ($p<0.05$) within 2-4 wk of storage time ca 0.5 log-cycle. The decreasing of viable counts of non-coated-immobilized of *B. longum* were not significant ($P>0.05$). The statistical analysis showed the significant effect ($p\leq0.05$) of storage time on the survival of free cells and non-coated-immobilized *B. bifidum* in Special Sacoo®SF-FDTB during storage in sterilized yogurt at 4-5°C for 4 wk (Figure 59). The viable counts of free cell and non-coated immobilized *B. bifidum* decreased ($p\leq0.05$) within 2-4 wk of storage time ca 0.8 log-cycle. The statistical analysis showed the significant effect ($p\leq0.05$) of storage time on the survival of free cells and non-coated-immobilized *B. infantis* in Special Sacoo®SF-FDTB during storage in sterilized yogurt at 4-5°C for 4 wk (Figure 60). The viable count of free cells of *B. infantis* decreased ($p\leq0.05$) within 3-4 wk of storage time ca 1 log-cycle while that of non coated-immobilized *B. infantis* showed the various values ca 0.3 log-cycle. However, the initial viable numbers of free cells and non-coated immobilized *B. longum, B. bifidum*, and *B. infantis* in Special Sacoo® SF-FDTB at 0 wk of storage time were different. Free cells had higher initial viable numbers. The interfering by anaerobic culture was not observed in sterilized yogurt. The viable counts of free cells of *B. longum*, *B. bifidum*, and *B. infantis* after storage in sterilized yogurt at 4-5°C for 4 wk were 9.37±0.15, 9.04±0.14, and 8.63±0.08 log CFU/mL, respectively. The viable counts of non-coated immobilized *B. longum*, *B. bifidum*, and *B. infantis* in Special Sacoo®SF-FDTB after storage in sterilized yogurt at 4-5°C for 4 wk were 9.02±0.07, 8.19±0.16, and 8.73±0.06 log CFU/mL, respectively. Sun and Griffiths (2000) reported that the yogurt starter cultures, and antibacterial metabolic products such as lactic acid, H_2O_2 and bacteriocin may affect the survival of bifidobacteria in yogurt. However, the survival of bifidobacteria in sterilized yogurt may not affect by the yogurt starter. Because sterilized yogurt was performed at 121°C for 15 min and this temperature could kill all bacteria and spore. There has not been the literature about the survival of immobilized bifidobacteria in sterilized yogurt.

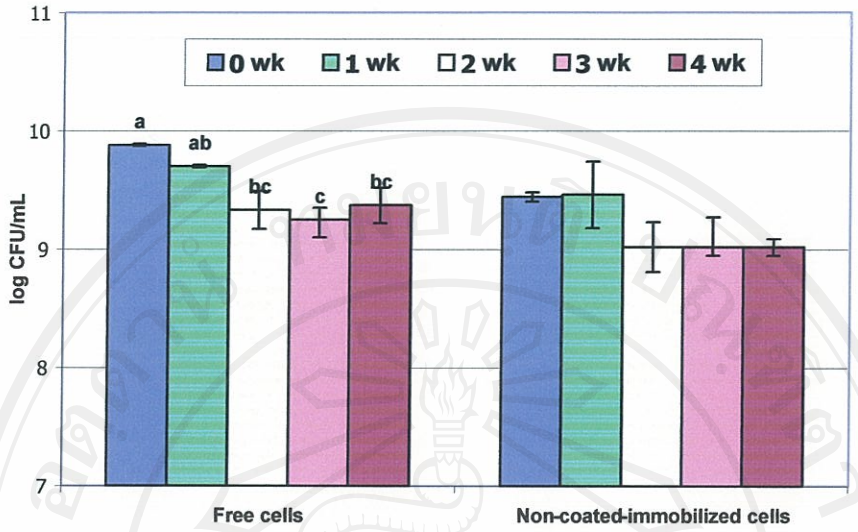

Figure 58 Effect of storage time on the survival of free cells and non-coated-immobilized of *B. longum* in Special Sacoo®SF-FDTB during storage in sterilized yogurt at 4-5°C for 4 wk.

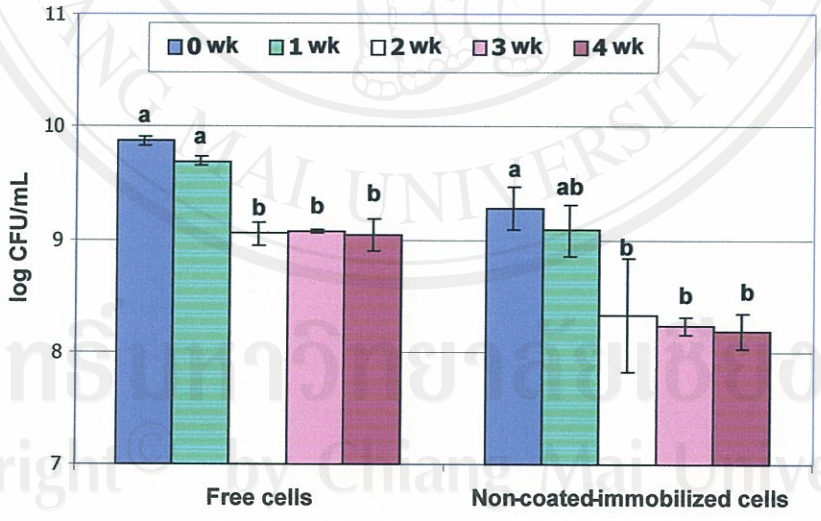

Figure 59 Effect of storage time on the survival of free cells and non-coated immobilized of *B. bifidum* in Special Sacoo®SF-FDTB during storage in sterilized yogurt at 4-5°C.

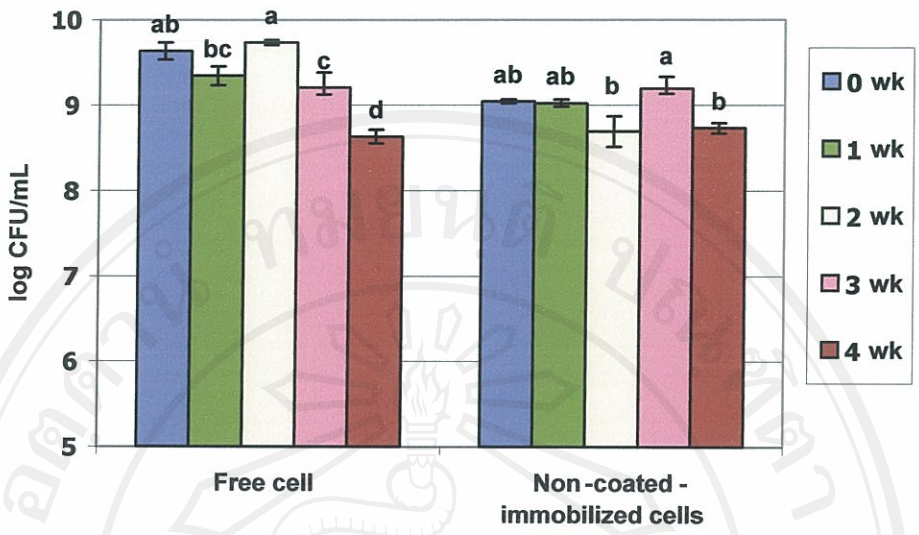

Figure 60 Effect of storage time on the survival of free cells and non-coated immobilized of *B. infantis* in Special Sacoo®SF-FDTB during storage in sterilized yogurt at 4-5°C.

4.9.1 Survival of free bifidobacterial cells and non-coated-immobilized *Bifidobacterium* spp. after incubated in simulated gastrointestinal fluids without enzyme at 37°C for 310 min

The survival of free cells and non-coated-immobilized *B. longum*, *B. bifidum*, and *B. infantis* in simulated gastrointestinal fluids were determined. The samples were collected from sterilized yogurt after storage at 4-5°C for 4 wk. The viable counts of non-coated-immobilized *B. longum* and *B. infantis* were higher than free cells ca 0.6 log CFU/mL. The viable counts of non-coated-immobilized *B. bifidum* was higher than free cells ca 0.2 log CFU/mL. The structure of Special Sacoo®SF-FDTB after storage in sterilized yogurt at 4-5°C for 4 wk may be weaken, and the protection property may decrease.

The viable counts of free cells and non coated-immobilized *B. longum*, *B. bifidum*, and *B. infantis* in Special Sacoo®SF-FDTB after storage in sterilized yogurt at 4-5°C for 4 wk remained high quantity at 8-9 log CFU/mL. To provide functional properties, the minimum level of viable bacteria should be approximately 10^6 bifidobacteria per mL of product at the time of consumption. The suggested therapeutic dose is 10^8–10^9 viable cells per day (Gardiner *et al.*, 2002). The viable counts of free cells and non-coated-immobilized *B. longum*, *B. bifidum*, and *B. infantis* in Special Sacoo®SF-FDTB after incubated in simulated gastrointestinal fluids decreased to 3-4 log CFU/mL. The results indicated that the tested bifidobacteria may survive during pass from the mouth through the human intestines. However, the *in vivo* experiment should be confirmed.

In conclusion, free cells and non-coated immobilized *B. longum*, *B. bifidum*, and *B. infantis* in Special Sacoo®SF-FDTB could survive in sterilized yogurt during storage time at 4-5°C for 4 wk and could survive after incubating in simulated gastrointestinal fluid without enzyme at 37°C for 310 min.

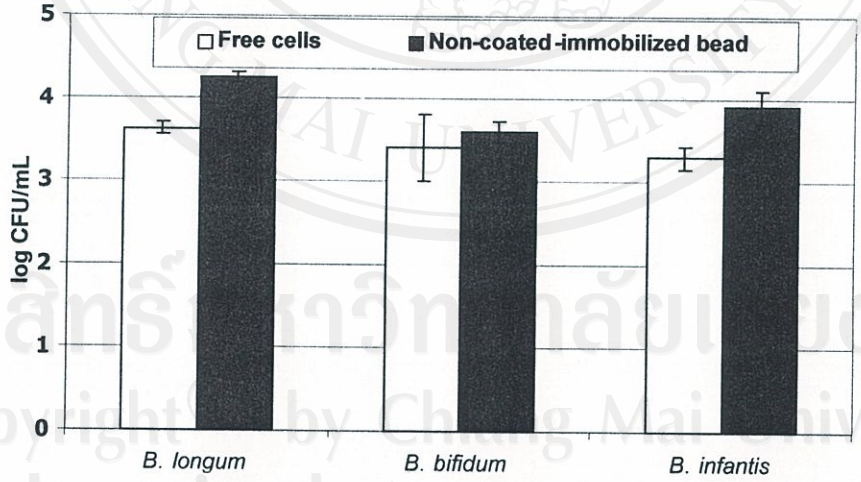

Figure 61 The survival of free cells, non-coated-immobilized *B. longum*, *B. bifidum*, and *B. infantis* in Special Sacoo®SF-FDTB after storage in sterilized yogurt at 4-5°C for 4 wk and incubated in simulated gastrointestinal fluids without enzyme at 37°C for 310 min.