Chapter 4

Result and Discussion

4.1 The effect of different types of bean milk on the survival of *L. acidophilus* during 15 days storage at 4°C

4.1.1 Chemical composition of bean milk

Different types of bean milk used in this research, including black bean, mung bean, red bean and soy bean milk were analyzed for their chemical composition as were exhibited in Table 4.1. From the Table, it showed clearly that all the bean milk contained moisture contents in the range of 91.00 ± 0.06 to 95.08 ± 0.01% (w/w). This high concentration of water was mainly due to the 8 hours soaking in water and grinded using distilled water at a ratio of 1:5 for the grain beans and distilled water, respectively (section 3.6.2). Among different types of bean milk, soy bean milk significantly had the highest total solid content, causing the milk to have significantly higher values of protein, fat, carbohydrate and ash compared to those of the other bean milk. The red bean milk was the milk with the highest moisture and fiber contents. For the black bean and mung bean milk, their nutritional values were shown to be similar, except for the fat content. The mung bean milk had a fat content 3 times more than that of the black bean milk.

Table 4.1 Chemical composition of bean milk

Types of bean milk	Chemical composition					
	Moisture (%)(w/w)	Protein (%)(w/w)	Fat (%)(w/w)	Fiber (%)(w/w)	Ash (%)(w/w)	Carbohydrate (%)(w/w)
Black bean	94.95± 0.23^{bc}	3.08±0.00^{b}	0.005±0.000^{a}	0.04±0.01^{a}	0.33±0.01^{a}	1.64±0.23^{a}
Mung bean	94.66±0.01^{b}	3.29±0.15^{b}	0.016±0.006^{a}	0.04±0.01^{a}	0.41±0.01^{b}	1.63±0.15^{a}
Red bean	95.08±0.01^{c}	2.45±0.02^{a}	0.059±0.007^{a}	0.12±0.03^{ab}	0.39±0.01^{b}	2.04±0.00^{a}
Soy bean	91.00±0.06^{a}	4.64±0.35^{c}	1.051±0.049^{b}	0.10±0.04^{b}	0.45±0.01^{c}	2.87±0.35^{b}

Values are means of two determination (n=2) with the standard deviation (±SD)

Common letters within a column are not significantly difference.

4.1.2 The survival of *L. acidophilus* during 15 days storage in bean milk at 4°C

This study was carried out to investigate the possibility of bean milk as a food vehicle for delivering *L. acidophilus* to customers that are interested with functional food products. To do this, an initial *L. acidophilus* population in the range of 8.66 ± 0.02 to 9.11 ± 0.06 log CFU/ml was inoculated into different types of bean milk, including black bean, mung bean, red bean and soy bean milk (Figure 4.1). A significant increase for up to 1.05 log CFU/ml of the *L. acidophilus* population was recorded during the storage period. Increasing population of the *L. acidophilus* in the bean milk could be due to nutrient compounds such as amino nitrogen contained in the bean milk. Charalampopoulos *et al.* (2003) presented that in order to evaluate the contribution of cereal constituents on probiotic cells survival, the individual effect of glucose, maltose and free amino nitrogen (FAN), which were added at concentrations that correlated to the reducing sugar and FAN contents of the cereal extracts, was examined. The survival of *L. acidophilus* increased by more than 1 log[10] cycle, even at very low concentrations of maltose and glucose (e.g., 0.67 g/l) and additions of tryptone and yeast extract, used as sources of FAN, enhanced *L. acidophilus* acid tolerance.

The high number of *L. acidophilus* population in the bean milk would comply with the minimum level of probiotic population to deliver its beneficial effect to the host as recommended by Ross *et al.* (2005). They recommended that the probiotic culture must be present in the product at minimum numbers of 10^7 CFU/ml or even higher numbers.

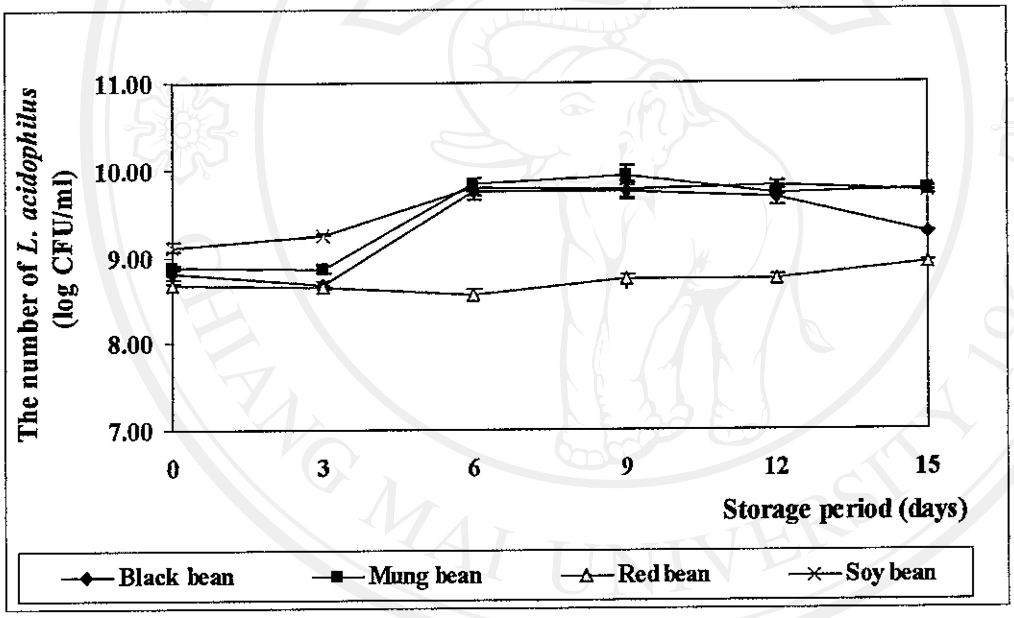


Figure 4.1 The number of *L. acidophilus* in different types of bean milk during 15 days storage at 4°C

To understand the effect of different types of bean milk in maintaining the viability of *L. acidophilus* during refrigerated storage, the viability slopes that linked all the *L. acidophilus* data during storage were calculated (Table 4.2). Results from this calculation confirmed the finding in Figure 4.1 that different types of bean milk did support the survival of *L. acidophilus* during low temperature storage. However, comparing different slope values displayed that the mung bean viability slope was significantly higher than those of the other types of bean milk. The lowest increasing rate of *L. acidophilus* population was found when the probiotic bacterium was inoculated in red bean milk. This finding could be due to nutrient compounds such as amino nitrogen contained in the bean milk, because the data in Table 4.1 showed that the protein content of the red bean milk was the lowest compared to the other bean milk. Because of this, the mung bean milk was chosen to be further studied in the next experiment.

Table 4.2 Viability slopes of different microorganism groups in different types of bean milk during 15 days storage at 4°C

Treatments	Culture media for different microorganism groups	
	MRS	PCA
Black bean	0.15 ± 0.02 ^{b}	0.15 ± 0.01 ^{b}
Mung bean	0.20 ± 0.01 ^{c}	0.20 ± 0.01 ^{c}
Red bean	0.05 ± 0.00 ^{a}	0.05 ± 0.00 ^{a}
Soya bean	0.14 ± 0.01 ^{b}	0.14 ± 0.01 ^{b}

Values are means of three determination (n=3) with the standard deviation (±SD).

Common letters within a column are not significantly difference.

In this research, the food carrier used to deliver *L. acidophilus* was pasteurized bean milk. Although the bean milk had been pasteurized, the media still had a high possibility to contain other microorganism types that survived heat treatments below 100°C (Anonymous, 2002b). To know the effect of these microorganisms on the viability of *L. acidophilus* in the bean milk during refrigerated storage, samples of the bean milk were subjected to total plate count analysis. Results of this analysis were exhibited in Figure 4.2 and Table 4.2.

Graphs in Figure 4.2 showed that different types of bean milk contained total microorganism population between 8.65 ± 0.05 to 9.09 ± 0.03 log CFU/ml at the beginning of the storage period. This high number of microorganisms in the bean milk suggested that the *L. acidophilus* was the dominant microorganism population grew on PCA medium, since the number was similar to the initial population of *L. acidophilus* found on the MRS medium. Beside that, the bean milk had passed a pasteurization process and should only contain low numbers of heat resistant microorganisms (Snyder, 1997). To confirm this conclusion some colonies that grown on the PCA medium were isolated, Gram stained and observed under a microscope. This observation displayed that the isolated colonies were Gram positive bacilli, which were typical colonies of *L. acidophilus* (Figure C2, Appendix C).

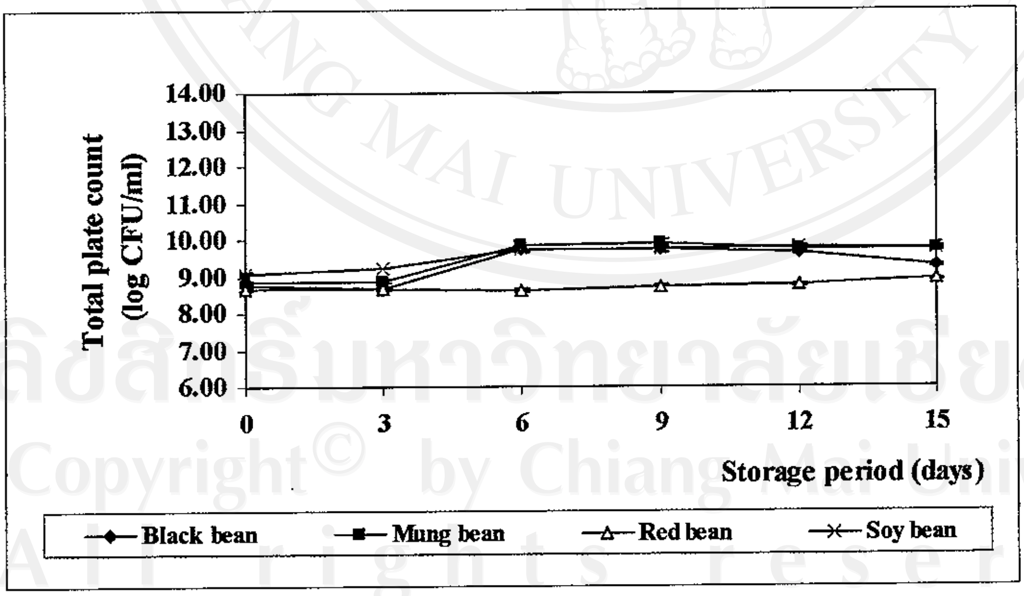


Figure 4.2 Total plate count of different types of bean milk added with *L. acidophilus* during 15 days storage at 4°C

During storage at 4°C, the number of total microorganisms in different types of bean milk could mainly be dominated by *L. acidophilus*. This could be seen from the Figures 4.1 and 4.2 and Table 4.2. Graph patterns in these Figures and viability slopes in the Table 4.2 indicated that other microorganisms that might survive the pasteurization process might not compete with the high population of *L. acidophilus* both in bean milk and microbiological media. However, bad odor that was detected at the end of the storage period suggested the possibility of the growth of other microorganisms in the bean milk.

4.1.3 Chemical properties of different types of bean milk added with *L. acidophilus* during 15 days storage at 4°C

4.1.3.1 Total soluble solid of different types of bean milk added with *L. acidophilus* during 15 days storage at 4°C

Total soluble solids of different types of bean milk were analyzed every 3 days during 15 days storage using a hand refractometer. Results of this analysis shown in Figure 4.3 and Table 4.3 displayed that different types of bean milk contained low levels of total soluble solids between 1.96 ± 0.09 to 2.38 ± 0.07°Brix at the beginning of the storage period. The highest total soluble solid content was found in soy bean milk (Figure 4.3).

During the storage period at chilled temperature, the total soluble solid levels of different types of bean milk did not demonstrate a significant change, except for the total soluble solid of the red bean milk (Figure 4.3 and Table 4.3). An increase for up to 0.43°Brix was recorded in these milk samples.

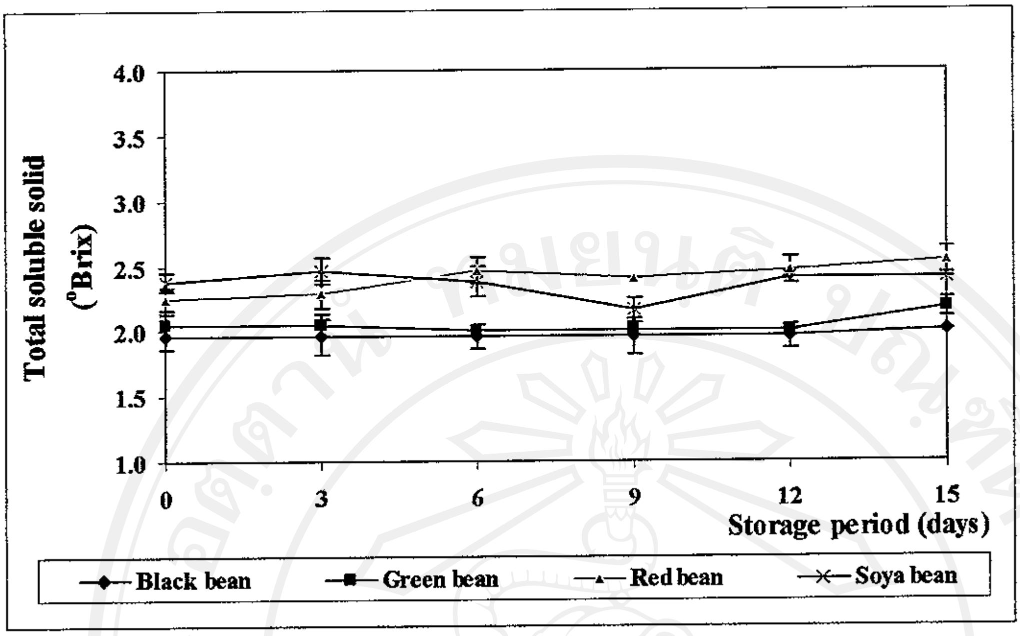


Figure 4.3 Total soluble solid (°Brix) of different types of bean milk during 15 days storage at 4°C

Table 4.3 Slopes for the chemical properties of different types of bean milk during 15 days storage at 4°C

Types of bean milk	Chemical properties		
	Total Soluble Solid	Total titratable acidity	pH
Black bean	0.01 ± 0.00^{b}	0.010 ± 0.001^{b}	-0.02 ± 0.00^{b}
Mung bean	0.02 ± 0.01^{b}	0.006 ± 0.001^{a}	-0.02 ± 0.01^{b}
Red bean	0.05 ± 0.01^{c}	0.009 ± 0.001^{b}	-0.02 ± 0.01^{b}
Soy bean	-0.01 ± 0.01^{a}	0.012 ± 0.001^{c}	-0.05 ± 0.02^{a}

Values are means of three determination (n=3) with the standard deviation (±SD).

Common letters within a column are not significantly difference.

4.1.3.2 Total titratable acidity of different types of bean milk added with *L. acidophilus* during 15 days storage at 4°C

Measurement results for total titratable acidities of different types of bean milk exhibited in Figure 4.4 and Table 4.3 showed that the total titratable acidity of different types of bean milk was not significantly different at the beginning of the storage period. Different types of bean milk had a total titratable acidity value between 0.07 ± 0.01 to 0.09 ± 0.01% lactic acid. During low temperature storage, the total titratable acidity levels of different types of bean milk were significantly increased at different increasing rates, except for the mung bean milk. An increase for up to 0.06% lactic acid could be recorded during the storage period for different types of bean milk (Figure 4.4).

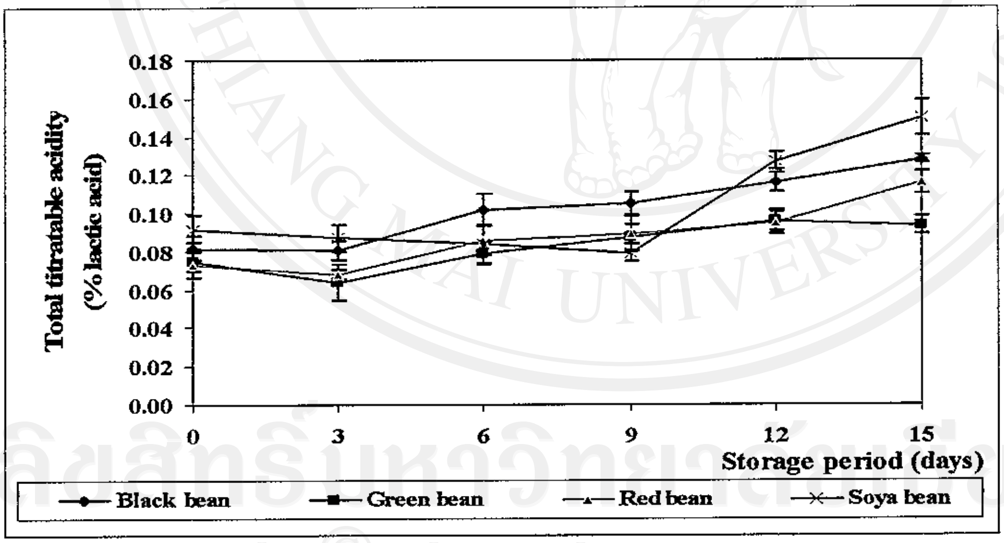


Figure 4.4 Total titratable acidity (% lactic acid) of different types of bean milk during 15 days storage at 4°C

The increase in total titratable acidity was mainly due to the metabolism activities of *L. acidophilus* that was detected to be significantly increased during the storage period (Figures 4.1 and 4.2 and Table 4.2). *L. acidophilus* is a lactic acid bacterium that produces lactic acid as its by-product (Anonymous, 2006b). However, it was interesting to point out that the lowest increasing rate for up to 0.02% lactic acid (Figure 4.4) in mung bean milk was accompanied by the highest increasing rate of *L. acidophilus* population, which was up to 1.05 log CFU/ml (Figure 4.1). On the other hand, the red bean milk that had an increase in total titratable acidity for up to 0.05% lactic acid could only have an increase in the *L. acidophilus* population for up to 0.26 log CFU/ml (Figure 4.1). This finding suggested that different nutrient compositions of different types of bean milk might affect the metabolism activities of *L. acidophilus* to produce different acid types. It could be suggested that to have a better understanding about this possibility, a further research about different by-products produced by *L. acidophilus* in different types of bean milk would need to be carried out. A review article, which assessed individual production of organic acids by *L. acidophilus* ATCC 4962 in the presence of mannitol, fructooligosaccharide (FOS) and inulin, showed that the production of individual organic acids was dependent on growth and the fermentability of prebiotics. Mannitol, FOS and inulin favoured the production of formic, lactic and butyric acids, respectively (Liong and Shah, 2005).

4.1.3.3 pH value of different types of bean milk added with *L. acidophilus* during 15 days storage at 4°C

Beside total titratable acidity, acidities of different types of bean milk were monitored every 3 days during 15 days storage using a pH meter. Results of this measurement displayed in Figure 4.5 and Table 4.3 demonstrated that different types of bean milk had a pH value range between 5.68 ± 0.06 to 6.09 ± 0.03 directly after the pasteurization processes. Between different types of bean milk, the soy bean milk significantly had the lowest pH value compared to those of the other bean milk. This could be due to different nutrient compositions of different types of bean milk, in which the soy bean milk had significantly the highest carbohydrate content compared to those of the other bean milk (Table 4.1). During 15 days storage at 4°C, the pH values of different types of bean milk were significantly reduced (Figure 4.5) confirming the finding of total titratable acidity (Figure 4.4). A similar explanation as for the results of the total titratable acidity could be seen in the previous section. The highest reduction rate of the soy bean milk pH values for -0.05±0.02 was consistent with the highest increasing rate of the milk total titratable acidities (Table 4.3). This finding indicated that the acidities of the soy bean milk needed to be considered if using the milk as a food carrier to deliver *L. acidophilus* and stored at 4°C for more than 15 days. Significant changes of the bean milk acidities during a storage period could raise customer objections to the sensory qualities of the products.

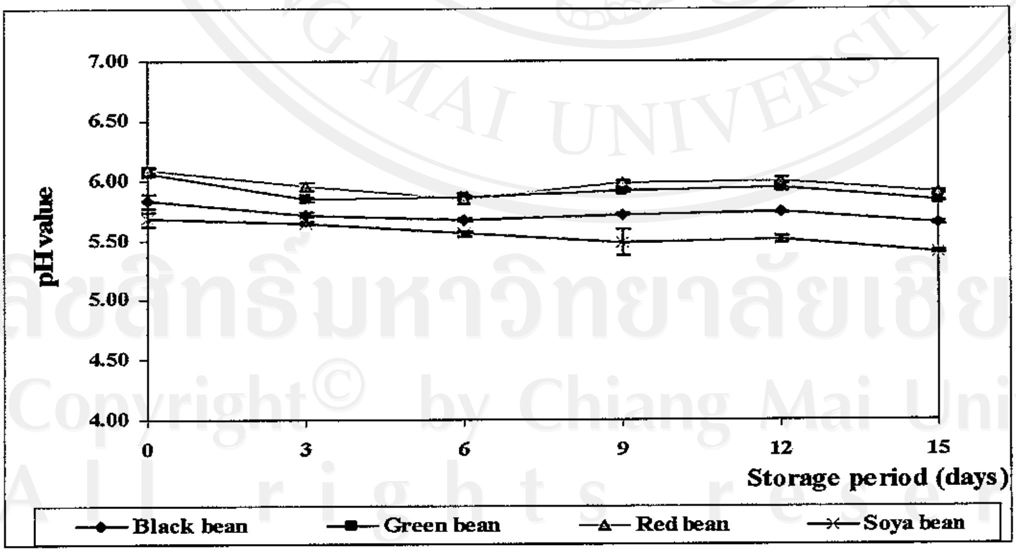


Figure 4.5 pH value of different types of bean milk during 15 days storage at 4°C

4.1.4 Physical properties of different types of bean milk added with *L. acidophilus* during 15 days storage at 4°C

4.1.4.1 Viscosities of different types of bean milk added with *L. acidophilus* during 15 days storage at 4°C

Monitoring for the viscosity of different types of bean milk that was conducted weekly during storage at 4°C could be seen in Tables 4.4 and 4.5. Collected data in Table 4.4 showed that different types of bean milk had significantly different viscosity values at the beginning of the storage period. The mung bean milk significantly had the lowest viscosity values compared to those of the other bean milk, while the black bean milk significantly exhibited the highest viscosity value. During the chilled storage temperature, the viscosities of different types of bean milk were significantly increased, except for the viscosity of the red bean milk. Increasing in the bean milk viscosities could be explained by the increasing numbers of *L. acidophilus* population as were shown in Figure 4.1. Measurement data demonstrated that the increasing viscosity rate of the mung bean milk, which was significantly the highest compared to those of the other bean milk (Table 4.5) also significantly had the highest increasing rate of the *L. acidophilus* population (Table 4.2). At the same time, the viscosity of the red bean milk that did not change significantly during the storage period had the lowest increasing rate of the *L. acidophilus* population (Table 4.2). This finding suggested that the metabolism activities of *L. acidophilus* could affect the viscosity of the bean milk. The possibility of the probiotic bacterium and/or other microorganisms producing exopolysaccharides (Anonymous, 2006c) should also not to be ruled out and counted in the consideration for the shelf life period of the bean milk kept at refrigerator temperature.

Table 4.4 Physical properties of different types of bean milk added with *L. acidophilus* during 15 days storage at 4°C

Types of bean milk	Storage (days)	Physical properties			
		Color values			Viscosity (cp)
		L*	a*	b*	
Black bean	0	65.56 ± 1.90 ^{a}	0.01 ± 0.12 ^{b}	3.05 ± 0.37 ^{a}	13.50 ± 0.95 ^{a}
	7	65.56 ± 0.51 ^{a}	0.04 ± 0.14 ^{c}	3.78 ± 0.24 ^{b}	12.27 ± 0.53 ^{a}
	14	66.13 ± 1.04 ^{b}	-0.03 + 0.03 ^{a}	4.19 ± 0.23 ^{c}	15.48 ± 0.68 ^{b}
Mung bean	0	66.03 ± 1.17 ^{b}	-4.44 + 0.49 ^{a}	8.81 ± 0.99 ^{a}	2.57 ± 0.02 ^{a}
	7	60.51 ± 9.02 ^{a}	-4.76 + 0.24 ^{a}	9.71 ± 0.62 ^{c}	14.74 ± 1.53 ^{b}
	14	64.77 ± 1.10 ^{b}	-4.08 + 0.31 ^{b}	9.25 ± 0.86 ^{b}	14.20 ± 0.92 ^{b}
Red bean	0	60.90 ± 0.19 ^{a}	1.27 ± 0.05 ^{a}	3.99 ± 0.16 ^{a}	6.93 ± 0.06 ^{ns}
	7	63.21 ± 0.30 ^{b}	1.18 ± 0.10 ^{b}	4.26 ± 0.27 ^{b}	7.00 ± 0.15 ^{ns}
	14	63.39 ± 0.17 ^{b}	1.05 ± 0.08 ^{c}	4.24 ± 0.12 ^{b}	6.88 ± 0.29 ^{ns}
Soy bean	0	83.08 ± 0.52 ^{a}	-0.59 + 0.06 ^{b}	11.20 ± 0.59 ^{a}	6.93 ± 0.09 ^{a}
	7	83.38 ± 0.71 ^{a}	-1.07 + 0.08 ^{a}	11.84 ± 0.37 ^{b}	7.50 ± 0.37 ^{a}
	14	84.13 ± 0.79 ^{b}	-1.19 + 0.11 ^{a}	11.71 ± 0.33 ^{b}	12.72 ±1.36 ^{b}

Values are means of three determination (n=3) with the standard deviation (±SD).

Common letters within a column and within each type of bean milk are not significantly difference.

4.1.4.2 Color values of different types of bean milk added with *L. acidophilus* during 15 days storage at 4°C

The color measurement of bean milk was carried out using a hunter color system and expressed in the color values of L*, a* and b*. The L* value measured the lightness of the bean milk sample and had a value between 0 to 100. The a* value had a color value between -60 to +60, in which the positive value indicated a red color whereas the negative value indicated a green color. The b* value also had a similar color value as the a* value with the positive value indicated a yellow color and the negative value correlated with a blue color (Anonymous, 2007f).

Table 4.5 Slopes for the physical properties of different types of bean milk added with *L. acidophilus* during 15 days storage at 4°C

Types of bean milk	Physical properties			
	Color values			Viscosity (cp)
	L*	a*	b*^{ns}	
Black bean	0.28 ± 0.27^{b}	-0.02 ± 0.05^{b}	0.57 ± 0.12	0.57 ± 0.12^{b}
Mung bean	-0.63 ± 0.69^{a}	-0.30 ± 0.21^{a}	0.62 ± 0.64	0.62 ± 0.64^{d}
Red bean	1.24 ± 0.06^{c}	-0.11 ± 0.02^{ab}	0.13 ± 0.10	0.13 ± 0.10^{a}
Soy bean	0.53 ± 0.28^{bc}	-0.30 ± 0.03^{a}	0.25 ± 0.27	0.25 ± 0.27^{c}

Values are means of three determination (n=3) with the standard deviation (±SD).

Common letters within a column are not significantly difference.

ns = not significantly difference.

The color of different types of bean milk displayed in Tables 4.4 and 4.5 clearly showed different color values between different bean milk types, which could be affected by different color compounds in different bean grains (Anonymous, 1995). At the beginning of the storage period, the soy bean milk significantly had the highest L* and b* values, which demonstrated that the bean milk was the whitest and had more yellow color compared to those of the other bean milk. At the same time, the red bean milk significantly exhibited the lowest L* value and the highest of the a* value (a positive value), which indicated that the bean milk was the darkest and had more red color compared to those of the other bean milk. The lowest a* value (a negative value) was found in the mung bean milk that suggested the bean milk significantly contained more green color compared to those of the other 3 bean milk. For the black bean milk, collected data only displayed that the milk had the lowest yellow color (Table 4.4).

During storage at 4°C, some of the bean milk color values were found to be significantly changed (Table 4.4). This included a significant increase in the L* value of the red bean milk and the b* value of the black bean milk and a significant decrease in the a* value of the red bean and soy bean milk samples. Some changes in the color values of the bean milk could be due to chemical reactions occurred at a slow rate at low storage temperature. Food and Agriculture Organization of the United Nations (Anonymous, 1995) presented that chemical reactions are responsible for changes in the color and flavour of foods during processing and storage. Color changes in food products were affected from pigments such as chlorophylls. Almost any type of food processing or storage causes some deterioration of the chlorophyll pigments. Phenophytinisation (with consequent formation of a dull olivebrown phenophytin) is the major change; this reaction is accelerated by heat and is acid catalysed. For anthocyanins, a water soluble pigment, the destruction rate of the pigment is pH dependent, being greater at higher pH values. Carotenoids, a group of mainly lipid soluble compounds responsible for many of the yellow and red colors of plant and animal products, is mainly degraded due to oxidation. The pigments may auto-oxidise by reaction with atmospheric oxygen at rates dependent on light and heat.

4.2 The effects of pH values and casein concentrations on the survival of *L. acidophilus* in mung bean milk during 15 days storage at 4°C

This section was used to have a better understanding about the survival of *L. acidophilus* in bean milk during chilled storage temperature. To do this, the result of the previous section of mung bean milk was applied as a food vehicle to keep the probiotic bacterium. The mung bean milk was chosen because the milk had shown to have the highest survival rate of *L. acidophilus* compared to those of the other bean milk (Figure 4.1 and Table 4.2). For the initial pH value, 3 different pH values of 6.0, 6.5 and 7.0 were used by adding 0.1 M sodium hydroxide into the mung bean milk. These pH values were chosen because optimal conditions for the growth and lactic acid production of *L. acidophilus* were pH 6.5 or 8.0 at 37°C (Tomas *et al.*, 2003). At the same time, 3 concentrations of casein, including 0, 125 and 250 mg/l, were added into the milk to understand whether the presence of the extra protein could give a better support for the viability of the probiotic bacterium. during the studied storage period (Sodini *et al.*, 2002). After mixed thoroughly, the mung bean milk was pasteurized at 69 ± 3°C for 30 minutes (Marshall and Arbuckle, 1996). cooled down and aseptically inoculated with *L. acidophilus*. The inoculated mung bean milk was then kept at 4°C for 15 days and analyzed regularly during the storage period.

4.2.1 The survival of *L. acidophilus* in mung bean milk affected by different pH values and casein concentrations during 15 days storage at 4°C

The viability monitoring of *L. acidophilus* in mung bean milk during 15 days storage at 4°C could be seen in Figure 4.6 and Table 4.6. Using an initial *L. acidophilus* population of 8.36 ± 0.04 to 8.81 ± 0.11 log CFU/ml, the probiotic bacterium showed a significant increase for its population number in every mung bean milk treatment during 15 days storage. An increase in the *L. acidophilus* population for up to 2.78 log CFU/ml was recorded when the bacterium was inoculated in the mung bean milk with an initial pH value of 6.5 and contained 250 mg/l casein. This finding was consistent with the result in the section 4.1.2, confirming the ability of the probiotic bacterium to use the nutrient compounds in the mung bean milk to maintain its survival rate at low storage temperature. However, it should be noted that the control mung bean milk (with an initial pH value of 6.0 and no addition of casein) had an increase in the *L. acidophilus* population for up to 2.4 log CFU/ml, which was an increase for more than 1.35 log CFU/ml compare to that of the finding in the section 4.1.2 for mung bean milk (Figure 4.1). This finding could be due to different production batch of mung bean grains used in different experimental section.

Comparing different initial pH values and casein concentrations, Figure 4.6 and Table 4.6 did not show any specific pattern that could be concluded, except for the initial pH of 6.5 that had higher increasing rates of *L. acidophilus* population with higher casein concentrations. This finding suggested that the effects of initial pH values and casein concentrations would be depended on the individual interactions of the two factors.

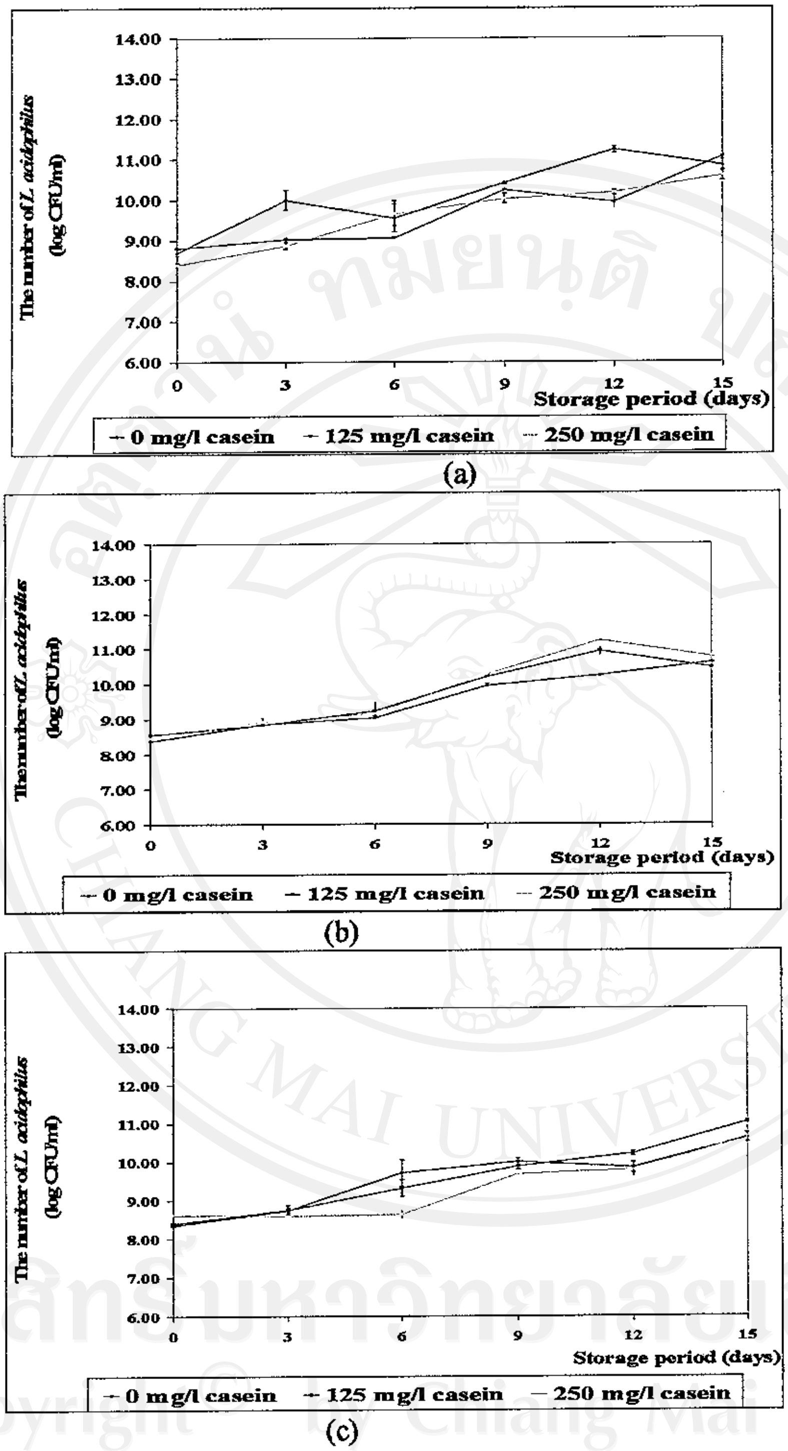


Figure 4.6 The number of *L. acidophilus* in mung bean milk affected by different pH values and casein concentrations during 15 days storage at 4°C. Different pH values of mung bean milk of 6.0 (a), 6.5 (b) and 7.0 (c).

Looking more detail into the collected data, it could be seen that higher increasing rates of the *L. acidophilus* population in different mung bean milk treatments compared to that of the control milk were only be significantly produced in the mung bean milk with an initial pH value of 6.5 and in the presence of 250 mg/l casein and in the mung bean milk with an initial pH value of 7.0 and added with 125 mg/l casein (Table 4.6). This result indicated that the two factors studied in this section still had a significant effect on the survival of *L. acidophilus* during low storage temperature. Since the mung bean milk with an initial pH value of 6.5 and added with 250 mg/l casein significantly showed the highest increasing rate of *L. acidophilus* population during the storage period, this condition was used to be studied further in the next experiment.

Table 4.6 Viability slopes of different microorganism groups in mung bean milk added with *L. acidophilus* and affected by different pH values and casein concentrations during 15 days storage at 4°C

Treatments		Culture media for different microorganism groups	
Initial pH value	Casein (mg/l)	MRS	PCA
6.0	0	0.44 ± 0.01^{abc}	0.44 ± 0.01^{bc}
	125	0.43 ± 0.04^{ab}	0.42 ± 0.00^{ab}
	250	0.44 ± 0.02^{abc}	0.41 ± 0.01^{a}
6.5	0	0.46 ± 0.00^{bc}	0.47 ± 0.00^{d}
	125	0.48 ± 0.02^{c}	0.51 ± 0.03^{e}
	250	0.56 ± 0.02^{e}	0.51 ± 0.03^{e}
7.0	0	0.43 ± 0.02^{ab}	0.40 ± 0.02^{a}
	125	0.52 ± 0.02^{d}	0.46 ± 0.02^{cd}
	250	0.42 ± 0.02^{a}	0.46 ± 0.00^{cd}

Values are means of three determination (n=3) with the standard deviation (±SD).
Common letters within a column are not significantly difference.

For the monitoring of total microorganisms in the mung bean milk on PCA medium. collected data in Figure 4.7 showed that the microorganism detected by the PCA medium had viability patterns like those of *L. acidophilus* measured using a MRS medium (Figure 4.6). This finding indicated that the *L. acidophilus* could be the main microorganism measured by the PCA medium as was explained in the section 4.1.2. However, details of the data indicated that the microorganism numbers grown on the PCA medium were generally lower than those of the *L. acidophilus* numbers grown on the MRS medium. This result could be due to the composition of different agar media, in which the PCA medium was designed to detect general viable microorganisms in dairy products, while the MRS medium was formulated to enumerate general lactic acid bacteria (Bridson, 1998). The presence of higher nutrient compounds, especially minerals, in the MRS medium could give a better support for the growth of *L. acidophilus*. Due to this reason, a slightly lower increase in the microorganism population for up to 2.76 log CFU/ml could be recorded from the enumeration results on the PCA medium. In addition, there was still a possibility that other microorganisms were also grown on the PCA medium during the storage period eventhough the colony appearances were not looked different by direct observation.

Between different treatments of mung bean milk, the total microorganism viability slopes of the mung bean milk that had an initial pH value of 6.5 were shown to be significantly higher than that of the control mung bean milk (Table 4.6). No specific conclusion for the effect of casein additions on the total microorganisms in the mung bean milk could be made.

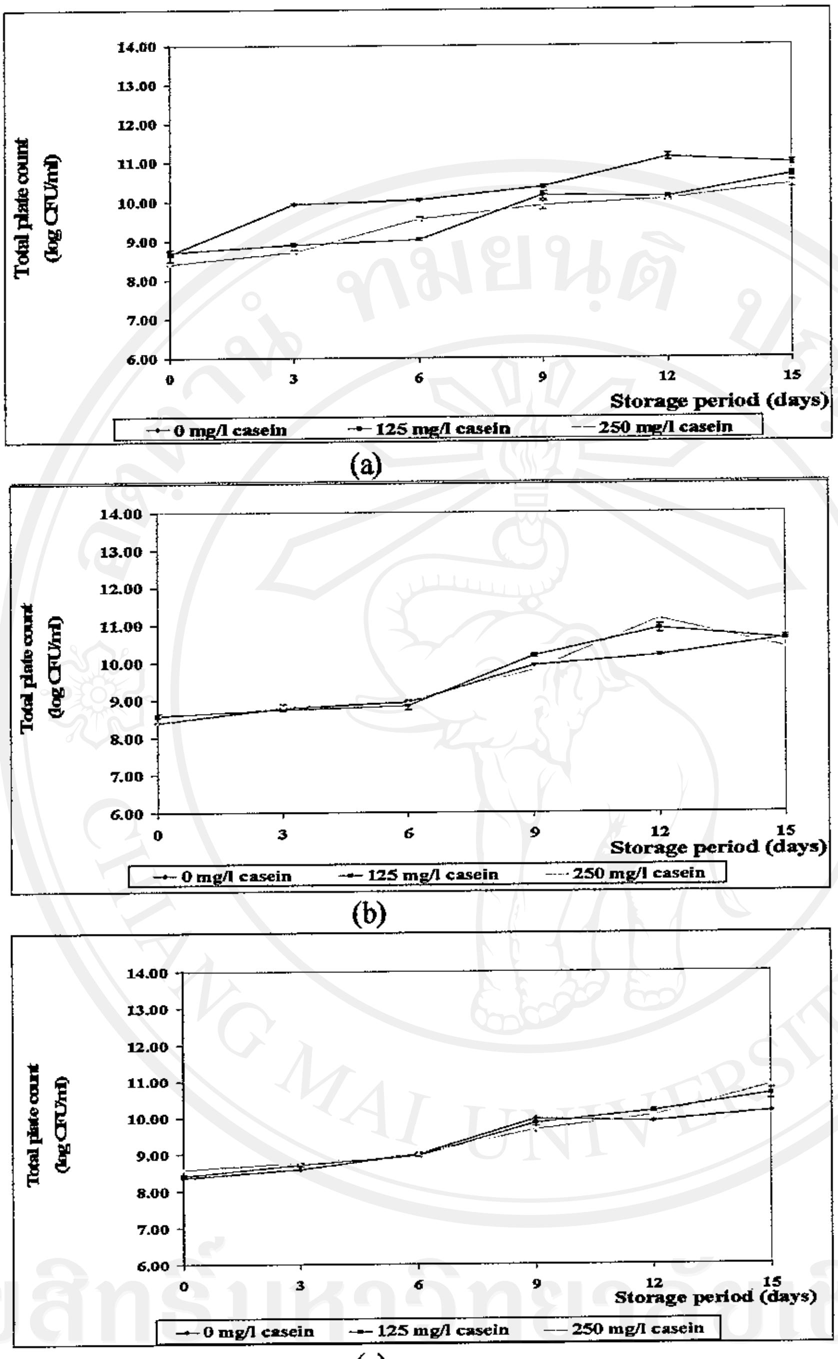


Figure 4.7 Total plate count of mung bean milk added with *L. acidophilus* and affected by different pH values and casein concentrations during 15 days storage at 4°C. Different pH values of mung bean milk of 6.0 (a), 6.5 (b) and 7.0 (c).

4.2.2 Chemical properties of mung bean milk added with *L. acidophilus* and affected by different pH values and casein concentrations during 15 days storage at 4°C

4.2.2.1 Total soluble solid of mung bean milk added with *L. acidophilus* and affected by different pH values and casein concentrations during 15 days storage at 4°C

Total soluble solid measurements of mung bean milk added with *L. acidophilus* were exhibited in Figure 4.8 and Table 4.7. Data from the Table showed that an addition of sodium hydroxide into the mung bean milk affected the total soluble solids of the milk. Significantly higher total soluble solid values were achieved in the mung bean milk with initial pH values of 6.5 and 7.0. The control mung bean milk (with an initial pH value of 6.0 and no addition of casein) had a total soluble solid content of 2.84 ± 0.09°Brix, which was significantly higher than that of the mung bean milk in the section 4.1.3.1 (Figure 4.3). The finding might reflect different chemical composition in different production batches of mung bean grains used in different experimental section.

During the storage period at 4°C, a significant reduction in the total soluble solid contents of different treatments of the mung bean milk could be seen in Figure 4.8 and Table 4.7. This reduction could mainly due to the increase in the *L. acidophilus* population during the storage period (Figure 4.6). However significant and higher reduction rates of the total soluble solids were found in the mung bean milk with initial pH values of 6.5 and 7.0 (Table 4.7).

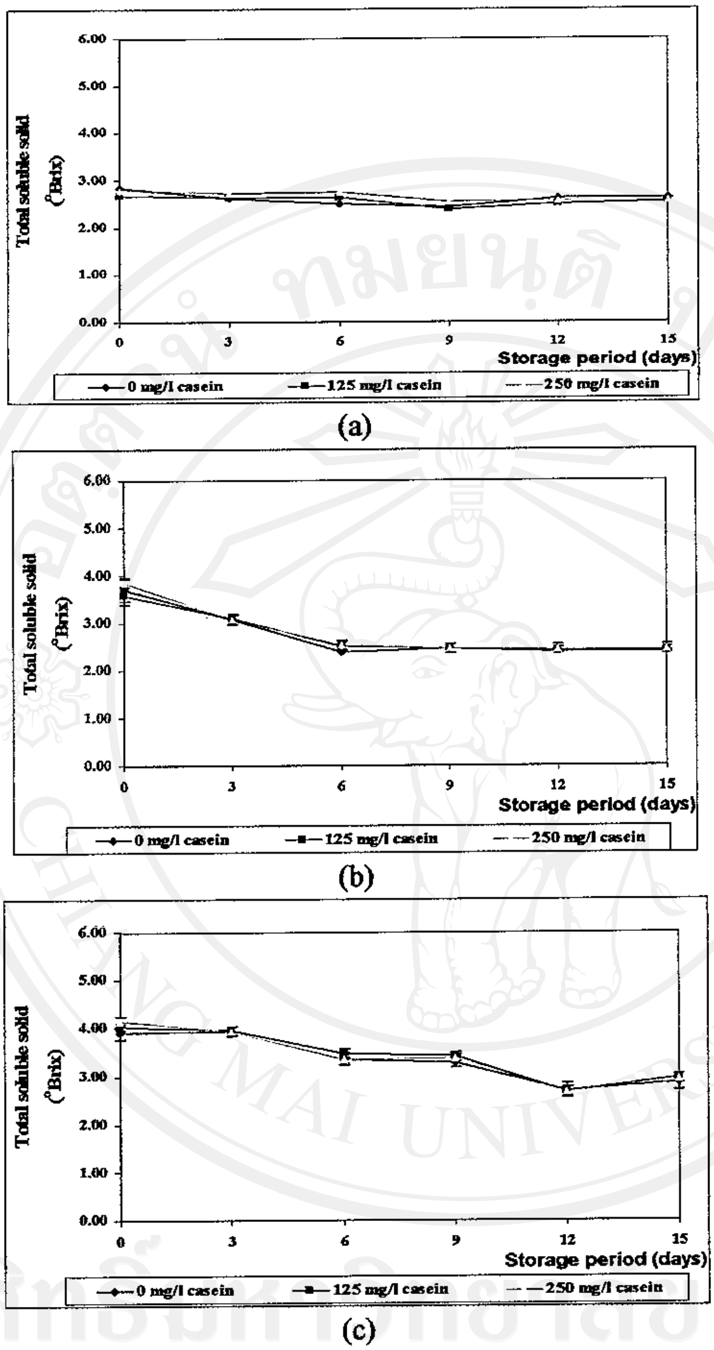


Figure 4.8 Total soluble solid (°Brix) of mung bean milk added with *L. acidophilus* and affected by different pH values and casein concentrations during 15 days storage at 4°C. Different pH values of mung bean milk of 6.0 (a), 6.5 (b) and 7.0 (c).

Since the increasing survival rates of the *L. acidophilus* were almost similar for all the mung bean milk treatments, except for the mung bean milk with a pH value of 6.5 and 250 mg/l casein and the milk with a pH value of 7.0 and 125 mg/l casein, the higher reduction rates of the total soluble solids in the mung bean milk with pH values of 6.5 and 7.0 could be due to the addition effect of sodium hydroxide.

Table 4.7 Slopes for the chemical properties of mung bean milk added with *L. acidophilus* and affected by different pH values and casein concentrations during 15 days storage at 4°C

Treatment		Chemical properties			
Initial pH value	Casein (mg/l)	Total soluble solid	Total titratable acidity	pH	Protein
6.0	0	-0.04 ± 0.01^{c}	0.006 ± 0.000^{b}	-0.08 ± 0.01^{b}	0.12 ± 0.08^{bc}
	125	-0.04 ± 0.01^{c}	0.008 ± 0.000^{c}	-0.08 ± 0.00^{bc}	-0.03 ± 0.14^{ab}
	250	-0.06 ± 0.03^{c}	0.009 ± 0.001^{c}	-0.10 ± 0.00^{a}	-0.03 ± 0.05^{ab}
6.5	0	-0.24 ± 0.05^{b}	0.005 ± 0.001^{a}	-0.07 ± 0.00^{d}	0.08 ± 0.03^{abc}
	125	-0.23 ± 0.01^{b}	0.004 ± 0.000^{a}	-0.07 ± 0.00^{cd}	0.18 ± 0.04^{c}
	250	-0.26 ± 0.01^{ab}	0.005 ± 0.000^{a}	-0.08 ± 0.00^{b}	-0.07 ± 0.02^{a}
7.0	0	-0.26 ± 0.03^{ab}	0.005 ± 0.000^{a}	-0.08 ± 0.00^{b}	0.04 ± 0.13^{abc}
	125	-0.26 ± 0.01^{ab}	0.004 ± 0.001^{a}	-0.08 ± 0.00^{b}	0.07 ± 0.12^{abc}
	250	-0.28 ± 0.02^{a}	0.004 ± 0.000^{a}	-0.08 ± 0.00^{b}	0.14 ± 0.03^{c}

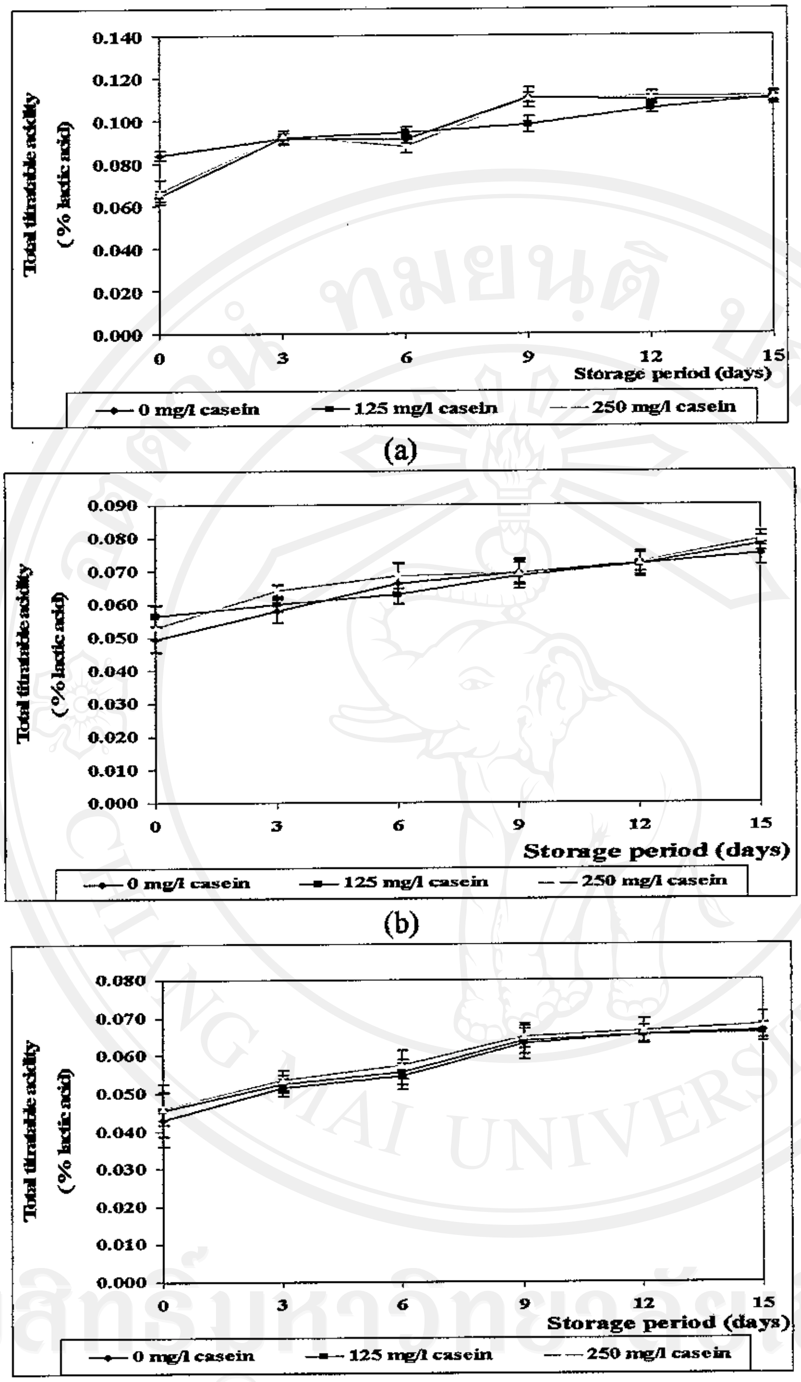
Values are means of three determination (n=3) with the standard deviation (±SD).

Common letters within a column are not significantly difference.

4.2.2.2 Total titratable acidity of mung bean milk added with *L. acidophilus* and affected by different pH values and casein concentrations during 15 days storage at 4°C

For the total titratable acidity measurement of the mung bean milk. the collected data displayed in Figure 4.9 and Table 4.7 showed that the initial total titratable acidities of the milk in this section were within the range of 0.04±0.01 to 0.08±0.00. Lower total titrable acidity values were found at higher pH values of the mung bean milk due to the addition of sodium hydroxide to adjust the mung bean milk pH values (section 3.6.4). The total titratable acidity of the control mung bean milk was found not to be significantly different to that of the mung bean milk in the section 4.1.3.2 (Figure 4.4).

Keeping the mung bean milk at 4°C caused a significant increase in the total titratable acidity values, which were up to 0.05% lactic acid. The main factor that contributed to the increase in the total titratable acidity values was the increase of the *L. acidophilus* population (Figure 4.6). However, it was interested to note that the increasing rate of the total titratable acidity was found to be significantly higher in the mung bean milk with a pH value of 6.0 compared to those of the mung bean milk with higher pH values (Table 4.7). This might indicate a higher metabolism activity of *L. acidophilus* at pH 6.0, eventhough this finding was not followed by a higher increasing rate of the *L. acidophilus* population (Table 4.6). At the same time. the total titratable acidity of the mung bean milk had a higher relationship with the milk pH value compared to the presence of casein in the milk.



(a)

(b)

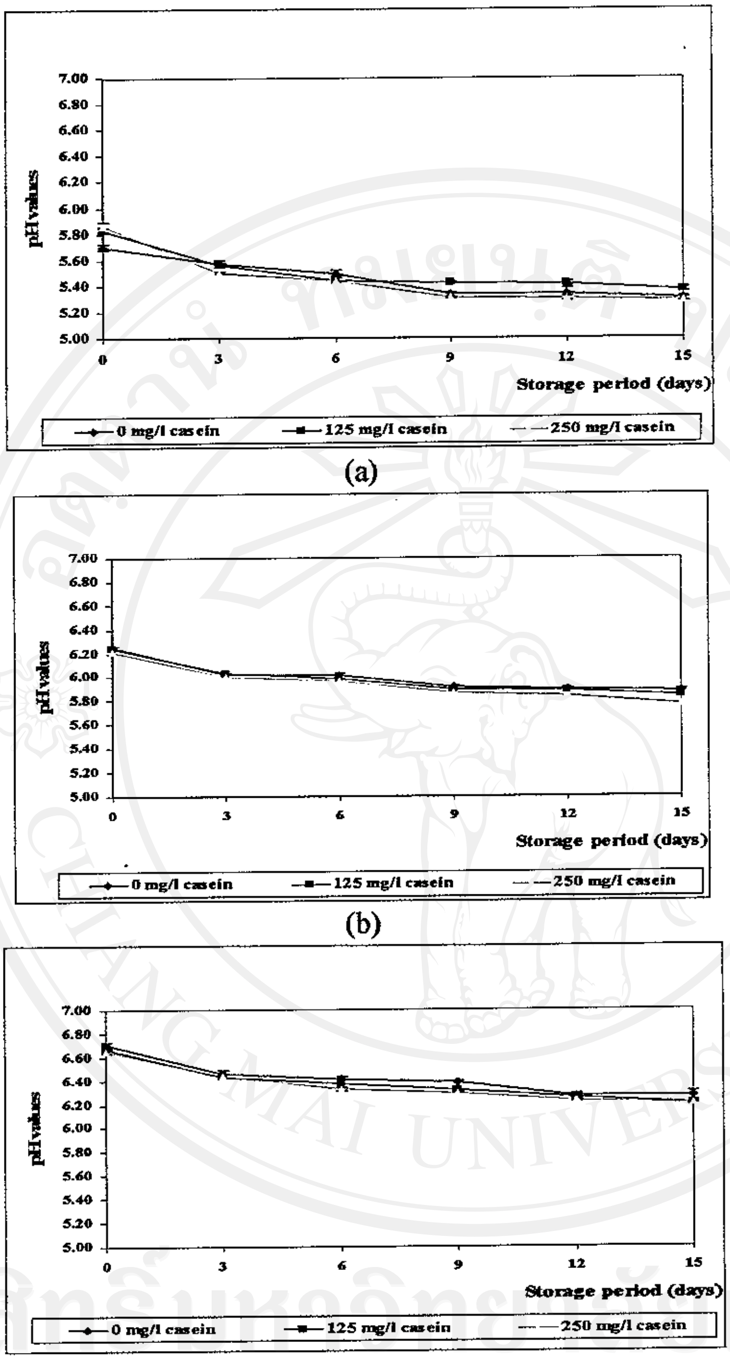
(c)

Figure 4.9 Total titratable acidity (% lactic acid) of mung bean milk added with *L. acidophilus* and affected by different pH values and casein concentrations during 15 days storage at 4°C. Different pH values of mung bean milk of 6.0 (a), 6.5 (b) and 7.0 (c).

4.2.2.3 pH value of mung bean milk added with *L. acidophilus* and affected by different pH values and casein concentrations during 15 days storage at 4°C

Another acidity parameter that was checked in the mung bean milks was pH values of the milk. The results of this measurement could be seen in Figure 4.10 and Table 4.7. At the beginning of the storage period, different treatments of the mung bean milk significantly had different pH values, which could be divided into 3 groups, due to the addition of sodium hydroxide (section 3.6.4). For the control mung bean milk, the initial pH value of the milk (5.71 ± 0.02) was found to be lower than that of the mung bean milk in the section 4.1.3.3 (Figure 4.5). This could be due to different production batches of mung bean grains that were also affected the results in the sections 4.2.1 and 4.2.2.1.

Monitoring of the pH values of the mung bean milk at 4°C clearly demonstrated significant reductions in the pH values of all the mung bean milk treatments (Figure 4.10 and Table 4.7). This finding supported the results of total titratable acidity measurement confirming that the increase in the *L. acidophilus* population was followed by an acidity development (Anonymous, 2006b). A pH value reduction between 0.37 to 0.58 was observed in different mung bean milk treatments. However, it was only the mung bean milk with a pH value of 6.0 and 250 mg/l casein that significantly had the highest reduction rate of the milk pH values (Table 4.7). Although this result was consistent with the total titratable acidity result, a high reduction rate of the pH value might affect the sensory and/or the physical properties of the mung bean milk product in the long term of storage period.



(a)

(b)

(c)

Figure 4.10 pH value of mung bean milk added with *L. acidophilus* and affected by different pH values and casein concentrations during 15 days storage at 4°C. Different pH values of mung bean milk of 6.0 (a), 6.5 (b) and 7.0 (c).

4.2.2.4 Protein contents of mung bean milk added with *L. acidophilus* and affected by different pH values and casein concentrations during 15 days storage at 4°C

Since in this experimental section casein was added as an extra protein source, the protein contents of the mung bean milk were checked weekly and exhibited in Figure 4.11 and Table 4.7. In general, the initial protein contents of different mung bean milk treatments were in the range of 2.70 ± 0.21 to 3.26 ± 0.09% (w/w). This result was consistent with the chemical composition of the mung bean milk discussed in the section 4.1.1 (Table 4.1). At the same time, different casein concentrations in the mung bean milk could not be significantly detected by the protein analysis used in this experiment (a total protein analysis by a Kjeldahl method) showing a low sensitivity detection of the applied method.

The protein contents of different mung bean milk treatments were slightly fluctuated during 15 days storage at 4°C (Figure 4.11 and Table 4.7). However, the changes were generally found not to be significantly different, eventhough there were significant increases in the *L. acidophilus* population (Figure 4.6) and in the mung bean milk acidities (Figure 4.9 and 4.10). More sensitive detection methods, such as electrophoresis or High Performance Liquid Chromatography, might need to be used to give a better understanding about the protein changes in the mung bean milk by *L. acidophilus* during refrigerated storage.

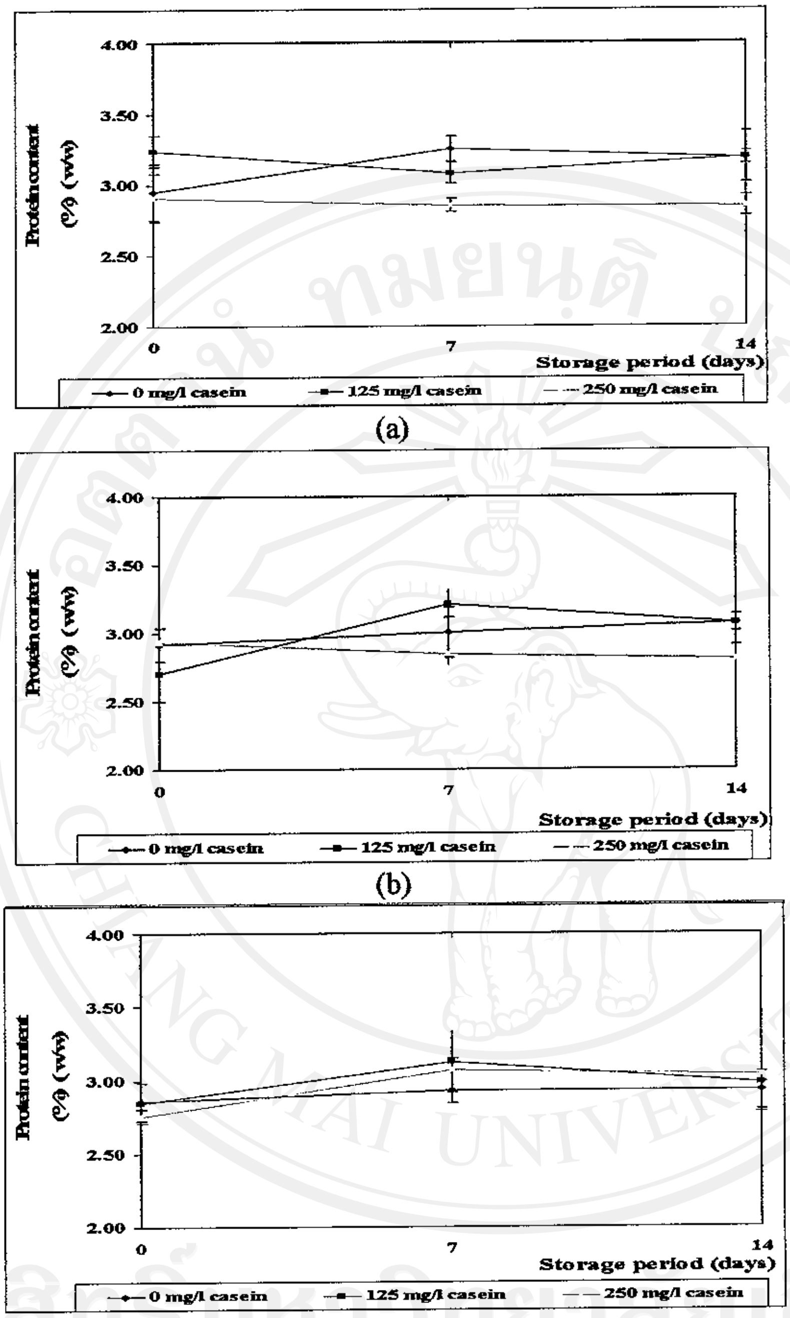


Figure 4.11 Protein content of mung bean milk added with *L. acidophilus* and affected by different pH values and casein concentrations during 15 days storage at 4°C. Different pH values of mung bean milk of 6.0 (a), 6.5 (b) and 7.0 (c).

4.2.3 Physical properties of mung bean milk added with *L. acidophilus* and affected by different pH values and casein concentrations during 15 days storage at 4°C

4.2.3.1 Viscosities of mung bean milk added with *L. acidophilus* and affected by different pH values and casein concentrations during 15 days storage at 4°C

Viscosities of different mung bean milk samples were measured weekly at 25 ± 2°C by a Brookfield viscometer using a needle number S18 and a viscometer speed at 150 rpm. The results of this analysis were shown in Table 4.8. Data from the Table demonstrated that the initial viscosity values of different mung bean milk samples were in the range of 2.93 ± 0.25 to 4.24 ±0.19. Higher viscosity values were found at higher initial pH values of the mung bean milk, which suggested that the addition of sodium hydroxide might affect the physical property of the bean milk. For the control mung bean milk, the initial viscosity value was also slightly but significantly higher than that of the mung bean milk in the section 4.1.4.1 (Table 4.4) showing the effect of different mung bean grain production batches in the different experimental section.

The viscosities of different mung bean milk samples were found to be significantly increased during the storage period (Tables 4.8 and 4.9), except for the control mung bean milk (an initial pH value of 6.0 and no casein addition) and the mung bean milk with an initial pH value of 6.0 and 125 mg/l casein. Changing in the mung bean milk viscosities could be due to the by-products of the *L. acidophilus* metabolism activities, such as exopolysaccharides, during the storage period. Higher increasing rates of viscosities were also observed at higher pH values, mainly 6.5 and 7.0, of the mung bean milk (Table 4.9).

Table 4.8 Physical properties of mung bean milk added with *L. acidophilus* and affected by different pH values and casein concentrations during 15 days storage at 4°C

Treatments		Storage period (days)	Physical properties			
			Color values			Viscosity (cp)
Initial pH value	Casein (mg/l)		L*	a*	b*	
6.0	0	0	67.33 ± 0.81^{b}	$-3.97 + 0.09^{a}$	11.95 ± 0.22^{a}	2.93 ± 0.25^{ns}
		7	65.61 ± 0.28^{a}	$-2.49 + 0.44^{b}$	12.45 ± 0.20^{b}	2.96 ± 0.09^{ns}
		14	68.48 ± 0.39^{b}	$-3.95 + 0.11^{a}$	12.35 ± 0.14^{b}	2.91 ± 0.11^{ns}
	125	0	68.25 ± 0.20^{b}	$-4.15 + 0.09^{a}$	11.61 ± 0.23^{a}	2.96 ± 0.17^{a}
		7	67.59 ± 0.19^{a}	$-2.98 + 0.02^{c}$	12.17 ± 0.03^{b}	3.02 ± 0.07^{b}
		14	68.68 ± 0.45^{b}	$-3.98 + 0.04^{b}$	12.10 ± 0.26^{b}	2.96 ± 0.14^{a}
	250	0	67.67 ± 0.91^{a}	$-3.47 + 0.26^{b}$	11.49 ± 0.34^{a}	3.33 ± 0.08^{a}
		7	67.86 ± 0.28^{a}	$-3.22 + 0.02^{b}$	12.70 ± 0.05^{b}	3.56 ± 0.08^{b}
		14	68.57 ± 0.69^{b}	$-4.1 + 0.08^{a}$	12.49 ± 0.25^{b}	3.70 ± 0.11^{c}
6.5	0	0	65.57 ± 0.60^{a}	$-3.05 + 0.16^{bc}$	9.30 ± 0.51^{a}	3.89 ± 0.04^{a}
		7	64.14 ± 0.18^{a}	$-2.78 + 0.03^{b}$	10.98 ± 0.04^{bc}	4.36 ± 0.29^{b}
		14	67.31 ± 0.50^{b}	$-3.76 + 0.06^{a}$	11.19 ± 0.17^{c}	5.71 ± 0.23^{c}
	125	0	65.22 ± 0.70^{a}	$-3.17 + 0.41^{b}$	9.41 ± 0.40^{a}	3.94 ± 0.11^{a}
		7	64.63 ± 0.42^{a}	$-2.45 + 0.17^{c}$	11.36 ± 0.15^{b}	4.50 ± 0.03^{b}
		14	67.38 ± 0.15^{b}	$-4.16 + 0.02^{a}$	11.33 ± 0.08^{b}	5.25 ± 0.33^{c}
	250	0	65.24 ± 1.79^{a}	$-4.14 + 0.46^{a}$	9.47 ± 0.28^{a}	$3.92 + 0.04^{a}$
		7	65.02 ± 0.05^{a}	$-2.71 + 0.03^{b}$	11.41 ± 0.05^{b}	$5.13 + 0.07^{b}$
		14	67.43 ± 0.34^{b}	$-4.06 + 0.14^{a}$	11.08 ± 0.06^{b}	$5.41 + 0.27^{b}$

Table 4.8 (Continued)

Treatments		Storage period (days)	Physical properties			
			Color values			Viscosity (cp)
Initial pH value	Casein (mg/l)		L*	a*	b*	
7.0	0	0	60.80 $\pm$ 0.31 ^{a}	-4.4 0+ 0.1 ^{a}	9.30 $\pm$ 0.16 ^{a}	3.98 $\pm$ 0.06 ^{a}
		7	61.32 $\pm$ 0.06 ^{a}	-3.65 + 0.03 ^{b}	10.01 $\pm$ 0.13 ^{b}	3.71$\pm$ 0.04 ^{a}
		14	63.38 $\pm$ 0.43 ^{b}	-3.87 + 0.09 ^{b}	9.51 $\pm$ 0.26 ^{a}	6.65 $\pm$ 0.40^{b}
	125	0	61.20 $\pm$ 0.18 ^{a}	-4.46 + 0.03 ^{a}	9.31 $\pm$ 0.06 ^{a}	4.21 + 0.17 ^{b}
		7	60.91 $\pm$ 0.10 ^{a}	-3.63 + 0.12 ^{b}	9.97 $\pm$ 0.30 ^{b}	3.78 + 0.05 ^{a}
		14	63.03 $\pm$ 0.46 ^{b}	-3.9 + 0.1 ^{b}	10.06 $\pm$ 0.34 ^{b}	7.46 + 0.37 ^{b}
	250	0	61.58 $\pm$ 0.15 ^{a}	-4.47 + 0.03 ^{a}	9.78 $\pm$ 0.03 ^{a}	4.24 $\pm$ 0.19 ^{b}
		7	61.32 $\pm$ 0.08 ^{a}	-3.6 + 0.01 ^{b}	10.34 $\pm$ 0.12 ^{b}	3.70 $\pm$ 0.05 ^{a}
		14	64.07 $\pm$ 0.17 ^{b}	-3.81 + 0.09 ^{b}	9.14 $\pm$ 0.13 ^{a}	7.37 $\pm$ 0.28 ^{c}

Values are means of three determination (n=3) with the standard deviation (±SD).

Common letters within a column and within each treatment of mung bean milk are not significantly difference.

ns = not significantly difference.

4.2.3.2 Color values of mung bean milk added with *L. acidophilus* and affected by different pH values and casein concentrations during 15 days storage at 4°C

For the color values of mung bean milk expressed as L*, a* and b* values, the collected data was displayed in Tables 4.8 and 4.9. This data showed that at the beginning of the storage period, different pH values of the mung bean milk significantly produced different color values, particularly for L* and b* values. The L* values were significantly higher (whiter color) at lower pH values of the mung bean milk, while the b* values were higher values (positive values, more yellow) in the mung bean milk with a pH value of 6.0 compared to those in the milk with pH values of 6.5 and 7.0. There was a possibility that the addition of sodium hydroxide affected these color values as were previously found for the total soluble solid and viscosity of the mung bean milk.

Table 4.9 Slopes for the physical properties of mung bean milk added with *L. acidophilus* and affected by different pH values and casein concentrations during 15 days storage at 4°C

Treatments		Physical properties			
		Color values			Viscosity
Initial pH value	Casein (mg/l)	L*	a*	b*	
6.0	0	0.57 ± 0.19^{abc}	0.01 ± 0.02^{c}	0.20 ± 0.06^{b}	0.09 ± 0.03^{ab}
	125	0.21 ± 0.15^{a}	0.09 ± 0.02^{c}	0.25 ± 0.09^{bc}	0.00 ± 0.06^{a}
	250	0.45 ± 0.57^{ab}	-0.32 ± 0.12^{b}	0.50 ± 0.07^{d}	0.22 ± 0.08^{b}
6.5	0	0.89 ± 0.23^{bcd}	-0.35 ± 0.06^{b}	0.94 ± 0.05^{e}	0.91 ± 0.09^{d}
	125	1.08 ± 0.42^{cd}	-0.50 ± 0.03^{a}	0.96 ± 0.13^{e}	0.66 ± 0.12^{c}
	250	1.09 ± 0.34^{cd}	0.04 ± 0.04^{c}	0.81 ± 0.05^{e}	0.74 ± 0.10^{cd}
7.0	0	1.29 ± 0.14^{d}	0.27 ± 0.03^{d}	0.10 ± 0.14^{b}	1.34 ± 0.10^{e}
	125	0.91 ± 0.19^{bcd}	0.28 ± 0.04^{d}	0.37 ± 0.11^{cd}	1.62 ± 0.19^{f}
	250	1.24 ± 0.03^{d}	0.33 ± 0.04^{d}	-0.31 ± 0.08^{a}	1.56 ± 0.19^{f}

Values are means of three determination (n=3) with the standard deviation (±SD).

Common letters within a column are not significantly difference.

Changing in the mung bean milk color values during storage at 4°C indicated that in general the L* values of the mung bean milk were increased. Higher and significant increasing rates of the L* values could be noted at higher pH values of the mung bean milk (Table 4.9). At the same time, there was not any specific patterns in the changes of the a* values. All the mung bean milk treatments contained low green color throughout the storage period. For the b* values, most of the mung bean milk samples significantly experienced increasing rates of the b* values (more yellow) during the storage period, except for the mung bean milk with a pH value of 7.0 and 250 mg/l casein. Changes in the mung bean milk color values could be due to the acidity development in the milk during 15 days at 4°C that affected the color compounds (Anonymous, 1995).

4.3 The effects of initial concentrations of *L. acidophilus* and an immobilized technique (an extrusion method) on the survival of the probiotic bacterium in mung bean milk during 15 days storage at 4°C

Although previous sections had shown that *L. acidophilus* could maintain its survival rate in mung bean milk during refrigerated storage for 15 days, in this section 2 factors of initial concentrations of the probiotic bacterium and an immobilization technique (an extrusion method) were further investigated to understand their effects on the viability of *L. acidophilus* in the mung bean milk. Two initial inoculation levels of *L. acidophilus* of 8 and 11 log CFU/ml combined with free or immobilized cells of *L. acidophilus* were added into pasteurized mung bean milk and stored of refrigerated temperature. A similar initial *L. acidophilus* population for free and immobilized cells could be achieved because the immobilized cells were prepared from young cultures of *L. acidophilus* cells with 1 log CFU/ml higher than the level used in the experiment (section 3.6.5.1). At the same time, the results of the previous section 4.2 were used in this section, including an initial pH value of 6.5 and 250 mg/l casein.

The immobilization technique applied in this section was an extrusion method that used a combination of calcium and alginate to form beads (Krasaekoopt *et al.*, 2004). This method was chosen because it is one of the techniques reported to enhance the survival of probiotic bacteria in dairy foods. Probiotic bacteria when encapsulated have acquired protection from stomach acidity and have increased their tolerance to bile. The viability of *Bifidobacterium pseudolongum* in simulated gastric juices was improved when it was encapsulated (Talwalkar and Kailasapathy, 2003).

The dimension of the *L. acidophilus* beads determined from 108 randomly selected beads were 2.01 ± 0.211 mm for the bead diameter and 562.68 mg for the mass of 108 beads or approximately 5.21 mg/bead. From these results, it was calculated the bead volume of 4.37 ± 1.35 mm^3 and a bead density of 1.32 mg/mm^3. The efficiency rate to release the *L. acidophilus* cells from the beads was 90.82 ± 2.83%.

4.3.1 The survival of *L. acidophilus* in mung bean milk affected by initial concentrations of the probiotic bacterium and an immobilization technique during 15 days storage at 4°C

The monitoring results for the survival of *L. acidophilus* in mung bean milk during low temperature storage could be seen in Figure 4.12 and Table 4.10. Data in the Figure showed that the initial inoculation level of 8 log CFU/ml *L. acidophilus* was carried out in the range of 8.07 ± 0.03 to 8.09 ± 0.01 log CFU/ml *L. acidophilus*, while the initial inoculation level of 11 log CFU/ml *L. acidophilus* was actually done in the range of 10.90 ± 0.04 to 10.92 ± 0.03 log CFU/ml. During the storage period, all the mung bean milk treatments demonstrated significant increases in the *L. acidophilus* population. An increase in the *L. acidophilus* population between 0.78 to 3.41 log CFU/ml could be recorded in this section (Figure 4.12). This result was consistent with the finding in the sections 4.1.2 and 4.2.1 confirming the ability of *L. acidophilus* to grow in the mung bean milk eventhough it was stored at low temperature.

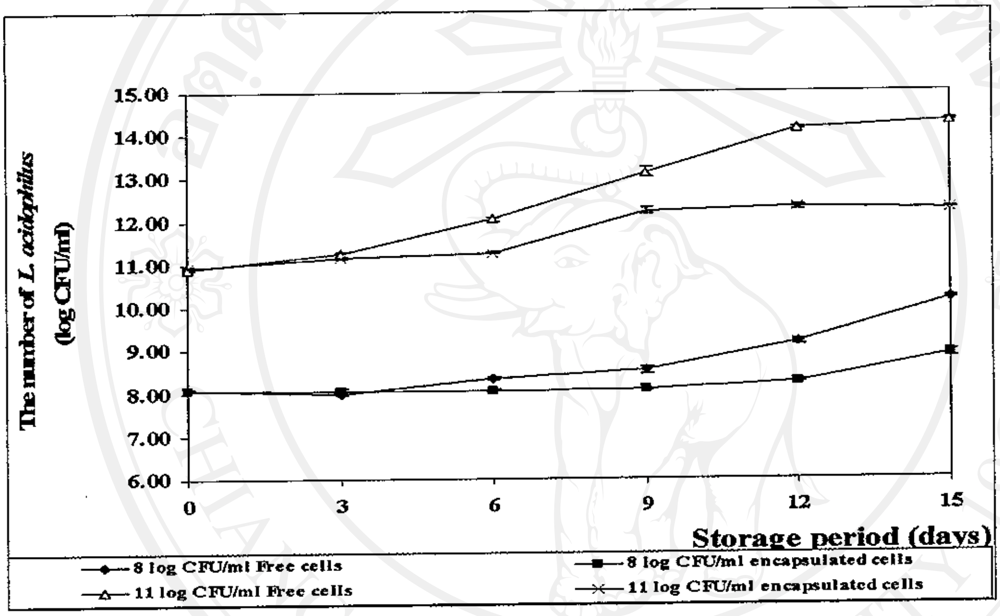


Figure 4.12 The number of *L. acidophilus* in mung bean milk affected by initial concentrations of the probiotic bacterium and an immobilization technique during 15 days storage at 4°C

Looking more details for the increasing rate of the *L. acidophilus* population, the calculation data in Table 4.10 showed clearly that higher initial inoculation levels of *L. acidophilus* both in free and encapsulated forms significantly produced higher increasing rates of the probiotic bacterium during the storage time. This result suggested that the initial inoculation level of *L. acidophilus* was an important factor in the production of *L. acidophilus*-added bean milk in order that the bean milk contained a specific level of the probiotic bacterium that could deliver its beneficial effect to the customer. Another conclusion that could be made from the data in Figure 4.12 and Table 4.10 was the free cells of *L. acidophilus* significantly had higher increasing population rates than that of the immobilized *L. acidophilus* cells. This finding could be due to different access mechanisms for the *L. acidophilus* cells to get the nutrient compounds from the mung bean milk. Since the immobilized/encapsulated cells of *L. acidophilus* were entrapped inside the bead matrix, the access of these cells to the nutrient compounds would be depended on the flow rate of the nutrient compounds from outside the beads to the inside. At the same time, the by-products from the *L. acidophilus* metabolism activities, such as organic acids and exopolysaccharides, would be depended on the flow rate of the by-products from inside the beads to the outside. A high accumulation of these by-products inside the beads might affect the survival of the *L. acidophilus* itself (Liong and Shah, 2005). At the same time, the free cells of *L. acidophilus* did not have any problem in accessing the nutrient compounds in the mung bean milk. Due to these factors, the increasing rate of the immobilized *L. acidophilus* cells was significantly lower than that of the free cells form. Although data in this study found that an immobilized form of a probiotic bacterium would produce a low increasing population rate of the bacterium during the storage period, the immobilized form could be useful for cases in which a high production of by-products by a probiotic bacterium significantly affected the chemical and physical properties of the food medium.

Table 4.10 Viability slopes of different microorganism groups in mung bean milk affected by initial concentrations of the *L. acidophilus* and an immobilization technique during 15 days storage at 4°C

Treatment of *L. acidophilus*	Culture media for different microorganism groups	
	MRS	PCA
8 log CFU/ml free cells	0.41 ± 0.01 [c]	0.41± 0.02 [c]
8 log CFU/ml encapsulated cells	0.13 ± 0.02 [a]	0.13± 0.01 [a]
11 log CFU/ml free cells	0.77 ± 0.01 [d]	0.76 ± 0.01 [d]
11 log CFU/ml encapsulated cells	0.32 ± 0.00 [b]	0.32 ± 0.01 [b]

Values are means of three determination (n=3) with the standard deviation (±SD).

Common letters within a column are not significantly difference

The results of the microorganism enumeration on PCA medium were displayed in Figure 4.13. The calculation for the microorganism viability patterns during the storage period could be seen in Table 4.10. In general, graphs in the Figure 4.13 clearly showed significant increases of the microorganism population detected on the PCA medium. The patterns of these graphs followed the graph patterns in Figure 4.12 indicating that the *L. acidophilus* could be the main microorganism detected on the PCA medium.

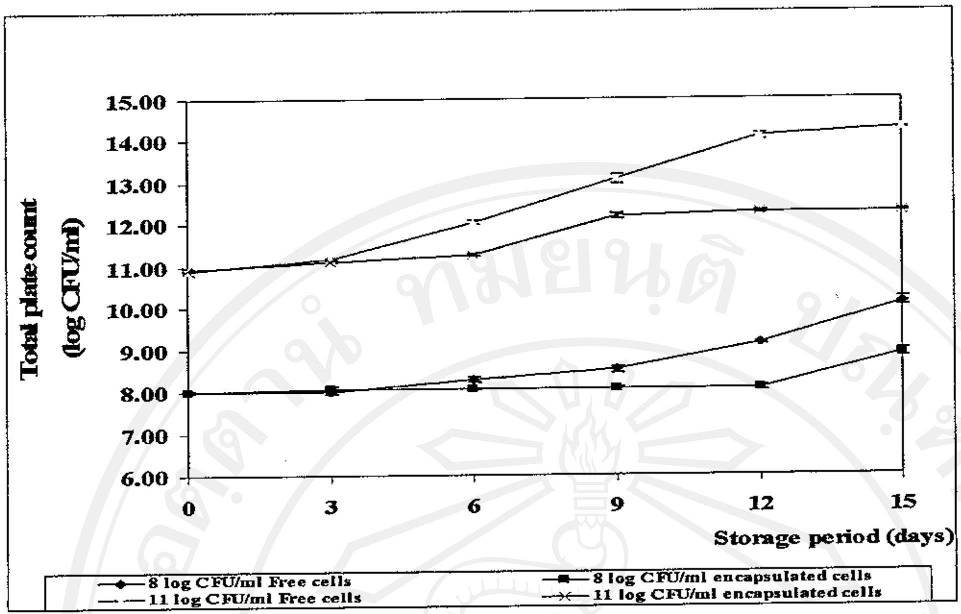


Figure 4.13 Total plate count of mung bean milk affected by initial concentrations of *L. acidophilus* and an immobilization technique during 15 days storage at 4°C

In addition, the calculation for the viability slopes of the microorganisms grown on the MRS and PCA media did not demonstrate significantly different values. Since the finding in this section was similar to the results in the sections 4.1.2 and 4.2.1, more details explanation could be read in these sections.

4.3.2 Chemical properties of mung bean milk added with *L. acidophilus* and affected by initial concentrations of the probiotic bacterium and an immobilization technique during 15 days storage at 4°C

4.3.2.1 Total soluble solid of mung bean milk added with *L. acidophilus* and affected by initial concentrations of the probiotic bacterium and an immobilization technique during 15 days storage at 4°C

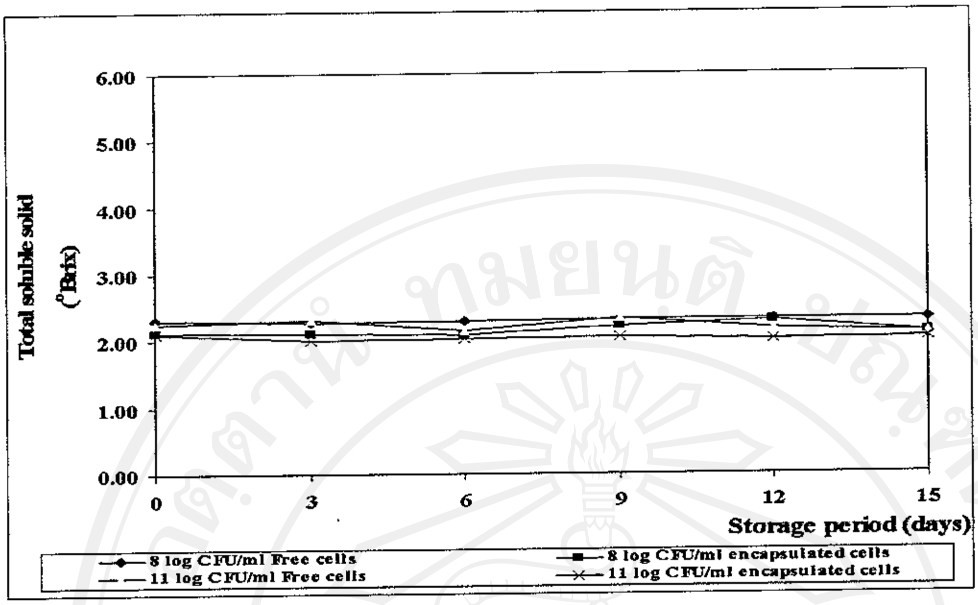


Figure 4.14 Total soluble solid (°Brix) of mung bean milk affected by initial concentrations of *L. acidophilus* and an immobilization technique during 15 days storage at 4°C

The total soluble solid results of different mung bean milk treatments were shown in Figure 4.14 and Table 4.11. Data in the Figure indicated that different inoculation levels and presentation form of *L. acidophilus* did not significantly affect the initial total soluble solid of the mung bean milk that had a value range of 2.11 ± 0.11 to 2.31 ± 0.15 °Brix. However, this value was significantly lower than that of the mung bean milk with a pH value of 6.5 and 250 mg/l casein (section 4.2.2.1) and slightly higher than that of the mung bean milk only (section 4.1.3.1). This could be due to different mung bean grain production batches and an addition of sodium hydroxide as were explained in the previous sections. During the storage period at 4°C, in general the total soluble solid of different mung bean milk treatments did not show significant changes between at the beginning and at the end of the storage period. However, decreasing rate of the total soluble solid contents of the mung bean samples could be observed as was shown by negative slope numbers in the Table 4.11. Although, the increasing *L. acidophilus* population in the mung bean milk (Figure 4.12) would contribute to the reduction in the total soluble solid in the mung bean milk samples, there was not any clear relationship between the two parameters.

Table 4.11 Slopes for the chemical properties of mung bean milk added with *L. acidophilus* and affected by initial concentrations of the probiotic bacterium and an immobilization technique during 15 days storage at 4°C

Treatment of *L. acidophilus*	Chemical properties		
	Total soluble solid	Total titratable acidity	pH
8 log CFU/ml free cells	0.00 ± 0.00^{bc}	0.001 ± 0.0003^{a}	-0.03 ± 0.00^{b}
8 log CFU/ml encapsulated cells	0.02 ± 0.01^{c}	0.001 ± 0.0002^{a}	-0.02 ± 0.00^{bc}
11 log CFU/ml free cells	-0.03 ± 0.02^{a}	0.004 ± 0.0001^{b}	-0.06 ± 0.01^{a}
11 log CFU/ml encapsulated cells	-0.01 ± 0.01^{ab}	0.001 ± 0.0003^{a}	-0.01 ± 0.01^{c}

Values are means of three determination (n=3) with the standard deviation (±SD).

Common letters within a column are not significantly difference.

4.3.2.2 Total titratable acidity of mung bean milk added with *L. acidophilus* and affected by initial concentrations of the probiotic bacterium and an immobilization technique during 15 days storage at 4°C

Figure 4.15 and Table 4.11 demonstrated the total titratable acidity of mung bean milk added with *L. acidophilus* during 15 days storage at 4°C. From the Figure, it could be seen clearly that the initial total titratable acidity of different mung bean milks treatments was within a range of 0.04 ± 0.002 to 0.08 ± 0.004% lactic acid, which was consistent with the result of the mung bean milk with a pH value of 6.5 and 250 mg/l casein in the section 4.2.2.2. However, a slightly but significantly higher acidity was displayed when a higher initial population of *L. acidophilus* was added into the bean milk.

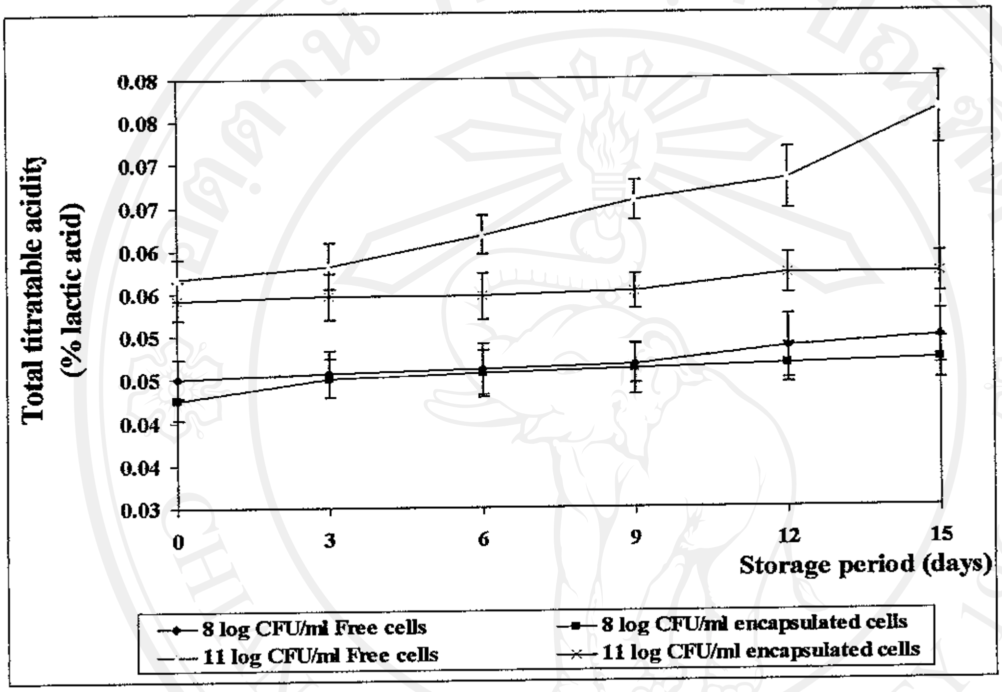


Figure 4.15 Total titratable acidity (% lactic acid) of mung bean milk affected by initial concentrations of *L. acidophilus* and an immobilization technique during 15 days storage at 4°C

Changes in the total titratable acidity of the mung bean milk during the storage period were found to be significantly increased, especially in the mung bean milk with 11 log CFU/ml free *L. acidophilus* cells. An increase between 0.003 to 0.019% lactic acid could be recorded from different mung bean milk treatments. This finding was mainly due to the increasing *L. acidophilus* population (Figure 4.12) and was consistent with the results in the sections 4.1.3.2 and 4.2.2.2.

4.2.2.3 pH value of mung bean milk added with *L. acidophilus* and affected by initial concentrations of the probiotic bacterium and an immobilization technique during 15 days storage at 4°C

Results for the monitoring of the mung bean milk pH values were exhibited in Figure 4.16 and Table 4.11. The pH values of different mung bean milk treatments were significantly different at the beginning of the storage period (Figure 4.16). Higher pH values were noticed at lower initial concentrations of *L. acidophilus*. At the same time, lower pH values could be recorded with free cells of *L. acidophilus* compared to those of the encapsulated cells.

Eventhough the initial pH values of different mung bean milk treatments were shown to be different, during the storage period the pH values of bean milk treatments were significantly decreased between a pH range of 0.04 to 0.27. Decreasing in the pH values found in this section confirmed the finding in the sections 4.1.3.3 and 4.2.2.3 and displayed its positive relationship with the presence of *L. acidophilus* in the milks. The highest pH reduction rate in the mung bean milk with 11 log CFU/ml free *L. acidophilus* cells (Table 4.11) was accompanied by the highest increasing rate of the *L. acidophilus* population in the milk (Table 4.10).

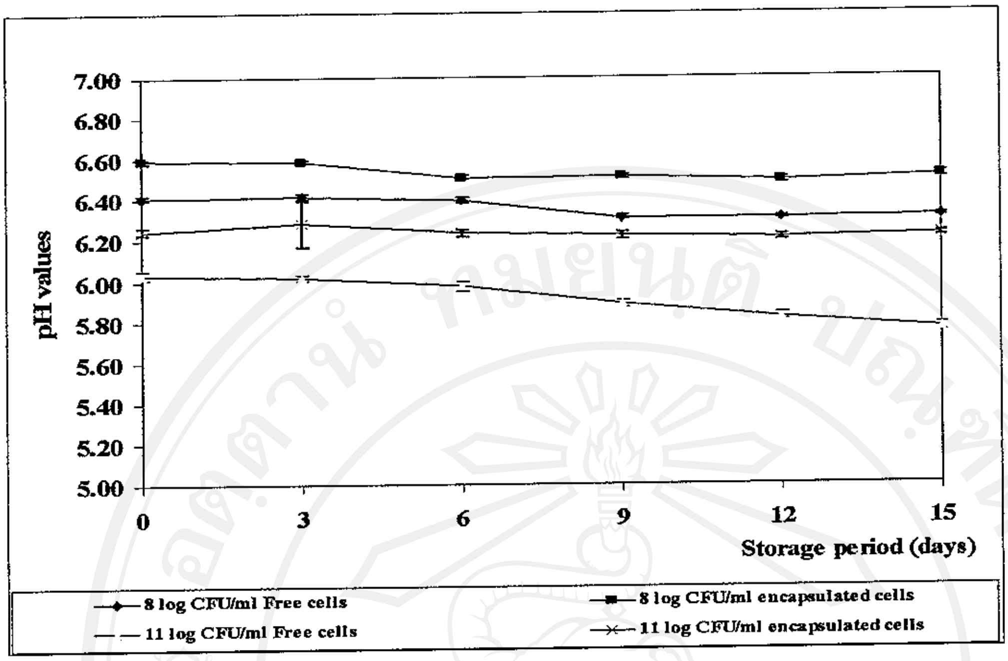


Figure 4.16 pH value of mung bean milk affected by initial concentrations of *L. acidophilus* and an immobilization technique during 15 days storage at 4°C

4.3.3 Physical properties of mung bean milk added with *L. acidophilus* and affected by initial concentrations of the probiotic bacterium and an immobilization technique during 15 days storage at 4°C

4.3.3.1 Viscosities of mung bean milk added with *L. acidophilus* and affected by initial concentrations of the probiotic bacterium and an immobilization technique during 15 days storage at 4°C

In this section, the viscosities of different mung bean milk treatments were measured by a Brookfield viscometer using a needle number S18 and two different viscometer speeds. A viscometer speed of 150 rpm was applied for the mung bean milk with 8 log CFU/ml *L. acidophilus* cells, while a speed of 40 rpm was used for the mung bean milk with 11 log CFU/ml *L. acidophilus* cells. Collected data shown in Table 4.12 clearly indicated that the initial viscosities of different mung bean milk samples were significantly different eventhough the viscosity of the mung bean milk with 8 log CFU/ml *L. acidophilus* free cells was not significantly different with that of the mung bean milk with a pH value of 6.5 and 250 mg/l casein in the previous section of 4.2.3.1. Different inoculation levels and presentation forms of *L. acidophilus* were significantly affected the viscosities of the mung bean milk. Higher viscosity values were found with higher levels of *L. acidophilus* inoculation levels, while lower viscosity values were recorded with the encapsulated cells of *L. acidophilus* compared to the free cells of the bacterium.

During the refrigerated storage for 15 days, all the treatments of the mung bean milk were shown to be significantly increased in their viscosity values (Table 4.12 and 4.13). Increasing viscosity rates of different mung bean milk treatments were found to have a strong relationship with the increasing population rates of the *L. acidophilus* (Table 4.10). Higher increasing viscosity rates (Table 4.13) were noticed with higher increasing *L. acidophilus* population rates (Table 4.10). This result suggested that the mass cells of the *L. acidophilus* and/or the by-products of the *L. acidophilus* metabolism activities could give a significant effect on the physical property of the food carrier that delivered the probiotic bacterium.

Table 4.12 Physical properties of mung bean milk added with *L. acidophilus* and affected by initial concentrations of the probiotic bacterium and an immobilization technique during 15 days storage at 4°C

Treatments of *L. acidophilus*	Storage period (days)	Physical properties			
		Color values			Viscosity (cp)
		L*	a*	b^{*ns}	
8 log CFU/ml free cells**	0	61.12 ± 0.27^{a}	$-5.92 + 0.12^{a}$	8.82 ± 0.40	3.81 ± 0.09^{a}
	7	61.62 ± 0.11^{a}	$-5.44 + 0.10^{a}$	8.82 ± 0.41	3.76 ± 0.36^{a}
	14	65.07 ± 0.68^{b}	$-3.75 + 0.11^{b}$	8.82 ± 0.42	11.99 ± 1.44^{b}
8 log CFU/ml encapsulated cells**	0	61.35 ± 0.54^{a}	$-5.68 + 0.10^{a}$	8.82 ± 0.43	3.70 ± 0.08^{a}
	7	63.19 ± 0.60^{b}	$-4.92 + 0.2^{b}$	8.82 ± 0.44	3.54 ± 0.33^{a}
	14	63.96 ± 0.60^{b}	$-5.15 + 0.35^{b}$	8.82 ± 0.45	4.75 ± 0.16^{b}
11 log CFU/ml free cells***	0	58.73 ± 2.49^{a}	$-3.92 + 0.35^{b}$	8.82 ± 0.46	43.74 ± 2.45^{a}
	7	59.40 ± 1.59^{b}	$-4.08+ 0.35^{a}$	8.82 ± 0.47	72.31 ± 1.29^{b}
	14	64.70 ± 2.37^{c}	$-4.07 + 0.31^{a}$	8.82 ± 0.48	74.99 ± 0.40^{b}
11 log CFU/ml encapsulated cells***	0	63.52 ± 1.21^{c}	$-4.11 + 0.23^{ab}$	8.82 ± 0.49	34.93 ± 0.23^{a}
	7	60.93 ± 2.14^{a}	$-4.03 + 0.42^{a}$	8.82 ± 0.50	51.36 ± 1.23^{b}
	14	62.83 ± 1.08^{b}	$-4.33 + 0.12^{b}$	8.82 ± 0.51	59.17 ± 5.60^{b}

Values are means of three determination (n=3) with the standard deviation (±SD).

Common letters within a column and within each *L. acidophilus* treatment are not significantly difference.

** viscosities (cp) of mung bean milk were measured at 150 rpm speed

*** viscosities (cp) of mung bean milk were measured at 40 rpm speed

ns = not significantly difference

Table 4.13 Slopes for the physical properties of mung bean milk added with *L. acidophilus* and affected by initial concentrations of the probiotic bacterium and an immobilization technique during 15 days storage at 4°C

Treatments of *L. acidophilus*	Physical properties			
	Color values			Viscosity
	L*	a*	b*	
8 log CFU/ml free cells	1.97 ± 0.42 ^{b}	1.09 ± 0.08 ^{c}	-0.61 ± 0.16 ^{ab}	4.09 ± 0.83 ^{b}
8 log CFU/ml encapsulated cells	1.31 ± 0.33 ^{ab}	0.26 ± 0.13 ^{b}	-0.97 ± 0.42 ^{a}	0.52 ± 0.08 ^{a}
11 log CFU/ml free cells	2.99 ± 1.89 ^{b}	-0.07 ± 0.16 ^{a}	-1.36 ± 1.09 ^{a}	15.62 ± 1.20 ^{d}
11 log CFU/ml encapsulated cells	-0.34 ± 0.61 ^{a}	-0.11 ± 0.10 ^{a}	0.54 ± 0.57 ^{b}	12.12 ± 2.25 ^{c}

Values are means of three determination (n=3) with the standard deviation (±SD).

Common letters within a column are not significantly difference.

4.3.3.2 Color values of mung bean milk added with *L. acidophilus* and affected by initial concentrations of the probiotic bacterium and an immobilization technique during 15 days storage at 4°C

Weekly observation for the color values of the mung bean milk samples could be seen in Tables 4.12 and 4.13. In general, different inoculation levels and presentation forms of *L. acidophilus* cells affected the initial L* and a* values. No significant differences of the initial b* value was observed with different treatments of the mung bean milk. The effect of the *L. acidophilus* cells on the L* and a* values was particularly pronounced at higher inoculation levels of the bacterium. This result suggested that eventhough scientific papers have recommended minimum levels of 6 log CFU/ml of a probiotic bacterium should be present in the food product at the end of its shelf life (Hou *et al.*, 2003), a too high inoculation level of the probiotic bacterium in the food product could also affect the color of the product.

Keeping the mung bean milk at 4°C for 15 days was mainly caused changes in the L* value of the mung bean milk samples. The changes in the a* values were only significantly recorded in the mung bean milk samples with 8 log CFU/ml free cells, while no significant changes could be noticed in the b* values of the mung bean milk samples throughout the storage period (Table 4.12). For the L* values, most of the mung bean milk samples showed significant increases in the value, except for the mung bean milk with 11 log CFU/ml encapsulated *L. acidophilus* cells. The highest increasing rate of the L* value was found in the mung bean milk with 11 log CFU/ml free *L. acidophilus* cells. This finding showed that a too high addition level of a probiotic bacterium might affect not only the initial color, particularly the L* value, of a food product, but also changes in the color of the food product during its storage period.

4.4 The survival of *L. acidophilus* in simulated gastrointestinal conditions during the shelf-life of the *L. acidophilus* in mung bean milk at 4°C for 15 days

From the probiotic definition, it was clearly stated that a microorganism would be categorized as a probiotic microorganism if the microorganism would remain viable during its transit in the gastrointestinal tract and could give a beneficial effect to the host through a variety of mechanisms (Hou *et al.*, 2003). To compile with this definition, it means that the probiotic microorganism needed to survive the high-acid gastric and bile-salt conditions in the stomach, in where the main digestion of food components takes places and in the intestines. This would include the *L. acidophilus* used in this research and added in the mung bean milk. Although *L. acidophilus* has been reported to be one of the probiotic bacteria that had a wide application in different commercial food products (Ross *et al.*, 2005), the ability of the *L. acidophilus* strain applied in this research needed to be checked *in vitro* for its ability to resist high-acid gastric and bile-salt conditions in the gastrointestinal tract. For the mung bean milk used in this experimental section, the results of the previous sections of 4.2 and 4.3, including a pH value of 6.5, 250 mg/l casein and 11 log CFU/ml free *L. acidophilus* cells, were also applied in the bean milk solutions that were utilized as a food vehicle to deliver the probiotic bacterium.

4.4.1 The survival of *L. acidophilus* in a simulated high-acid gastric condition during the shelf-life of the *L. acidophilus* in mung bean milk at 4°C for 15 days

The simulated high-acid gastric conditions exercised in this experimental sections were pH values of 1.5, 2.0 and 2.5 to represent high pH values in the stomach that has been reported to have a pH value range between 1 to 2 (Anonymous. 2003). For a control, a pH value of 6.0 was applied and all the treatments used a MRS broth as a tested medium and an incubation period for 3 hours at 37°C. The survival results of the *L. acidophilus* in simulated high-acid gastric conditions were exhibited in Tables 4.14 and 4.15. The collected data in Table 4.14 showed clearly that the *L. acidophilus* that had a good survival rate in mung bean milk samples (sections 4.2.1

and 4.3.1) produced a different survival data at higher pH values of the MRS broth. On the 0 day of the mung bean milk storage period, a high number of *L. acidophilus* could be detected in the control MRS broth (pH 6.0) immediately after the inoculation and was significantly increased during the incubation period for 3 hours at 37°C.

Table 4.14 The survival of *L. acidophilus* in simulated high-acid gastric conditions during the shelf-life of the *L. acidophilus* in mung bean milk at 4°C for 15 days

Storage period of *L. acidophilus* in mung bean milk (days)	pH values of MRS broth	*L. acidophilus* (log CFU/ml)			
		Incubation times in the MRS broths			
		0	1	2	3
0	1.5	No growth	No growth	No growth	No growth
	2.0	8.09 ± 0.05	No growth	No growth	No growth
	2.5	8.05 ± 0.06	No growth	No growth	No growth
	6.0	10.68 ± 0.05	10.71 ± 0.12	10.80 ± 0.08	11.15 ± 0.02
7	1.5	No growth	No growth	No growth	No growth
	2.0	8.26 ± 0.02	No growth	No growth	No growth
	2.5	8.26 ± 0.01	No growth	No growth	No growth
	6.0	11.89 ± 0.02	11.89 ± 0.01	12.00 ± 0.09	12.21 ± 0.01
14	1.5	No growth	No growth	No growth	No growth
	2.0	9.00 ± 0.01	No growth	No growth	No growth
	2.5	8.96 ± 0.04	No growth	No growth	No growth
	6.0	13.19 ± 0.05	13.20 ± 0.01	13.83 ± 0.06	13.96 ± 0.03

Values are means of three determination (n=3) with the standard deviation (±SD).

However at higher pH values of 2.0 and 2.5, the population of *L. acidophilus* was significantly reduced for up to 2.63 log CFU/ml immediately after the inoculation of the culture to the MRS broth. A very low number of *L. acidophilus* (<1 log CFU/ml) could be detected after the bacterium was incubated for 1 hour at 37°C in the MRS broths with pH values of 2.0 or 2.5 or directly after the *L. acidophilus* was inoculated in the MRS broth at a pH value of 1.5. The finding suggested that the *L. acidophilus* strain that used in this research might not be able to survive the high-acid gastric condition in the stomach and might not fulfill the specific characteristics of the probiotic microorganisms.

During the storage period of the mung bean milk samples at 4°C, a significant increasing rate of *L. acidophilus* in the control MRS broth (pH 6.0) could be observed. The bacterium could also be significantly increased during 3 hours incubation period at 37°C in the MRS broth (Tables 4.14 and 4.15). Interestingly, a higher survival rate of *L. acidophilus* in the control MRS broth (pH 6.0) was not followed by a better resistant of the bacterium at higher pH values. Higher decreasing rates of the *L. acidophilus* population immediately after being inoculated in the MRS broths with pH values of 2.0 and 2.5 could be recorded. Reduction in the number of *L. acidophilus* for up to 3.63 and 4.23 log CFU/ml could be noticed when the bacterium was added in the MRS broth at higher pH values after being storage for 7 and 14 days, respectively, in the mung bean milk. At the same time, the *L. acidophilus* was not be able to survive better in the MRS broth at a pH value of 1.5. This result showed that eventhough the *L. acidophilus* population could increase during the storage period of the bacterium in the mung bean milk, only some from this increasing population that had a good resistant in an adverse environment. Beside that, if the initial *L. acidophilus* population could not survive at pH 1.5, keeping the bacterium in the mung bean milk that had a pH value of 6.5 would not improve its resistant characteristic at high pH values, especially at pH 1.5.

Table 4.15 The survival slopes of *L. acidophilus* in simulated high-acid gastric conditions during the shelf-life of the *L. acidophilus* in mung bean milk at 4°C for 15 days

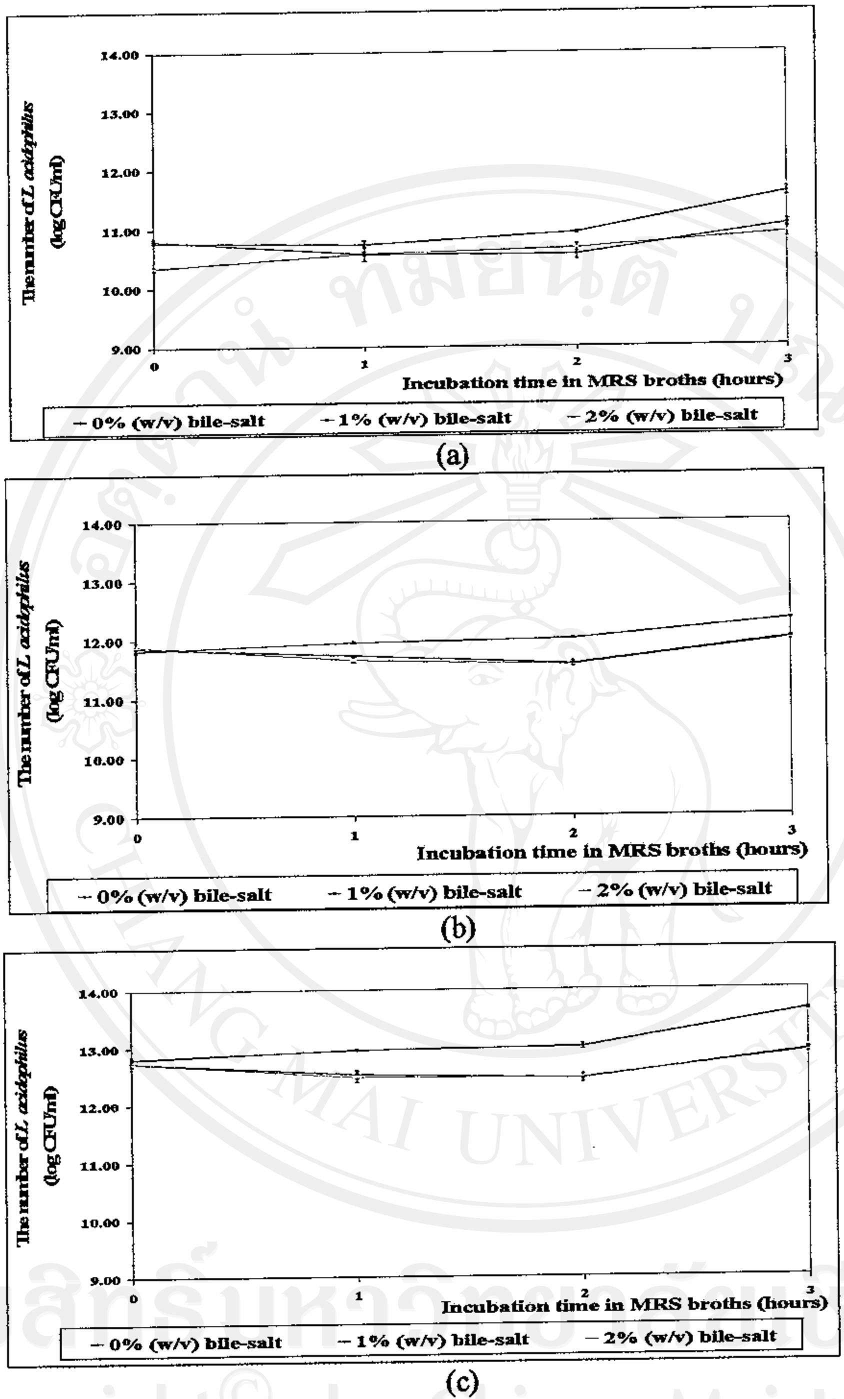
Storage period of *L. acidophilus* in mung bean milk (days)	pH values of MRS broth	The survival slopes of *L. acidophilus*
0	1.5	-
	2.0	-
	2.5	-
	6.0	0.15 ± 0.03^{a}
7	1.5	-
	2.0	-
	2.5	-
	6.0	0.11 ± 0.01^{a}
14	1.5	-
	2.0	-
	2.5	-
	6.0	0.29 ± 0.02^{b}

Values are means of three determination (n=3) with the standard deviation (±SD).

Common letters within a column are not significantly difference.

4.4.2 The survival of *L. acidophilus* and in a simulated bile-salt condition during the shelf-life of the *L. acidophilus* in mung bean milk at 4°C for 15 days

In the stomach beside a high-acid gastric condition, food components would also experience a bile-salt situation, in which enzymes would digest the components into smaller units to facilitate the adsorption process by the intestines. This process would also be faced by the *L. acidophilus* when the probiotic bacterium was consumed and passed the host intestines.



(a)

(b)

(c)

Figure 4.17 The survival of *L. acidophilus* in simulated bile-salt conditions during the shelf-life of the *L. acidophilus* in mung bean milk at 4°C for 15 days. The storage period of the *L. acidophilus* in mung bean milk at 0 (a), 7 days (b) and 14 days (c).

To understand the resistant of the bacterium in the bile-salt condition, the bacterium was inoculated in the MRS broths with different bile-salt concentrations, including 0, 1 and 2% (w/v). The results of the *L. acidophilus* enumeration in different bile-salt conditions during 3 hours incubation period at 37°C were shown in Figure 4.17 and Table 4.16. The graph in the Figure 4.17a clearly displayed that the *L. acidophilus* strain used in this research was resistant to the bile-salt conditions at the beginning of the mung bean milk storage period. At different bile-salt concentrations, the *L. acidophilus* was only found to be significantly reduced at 2% (w/v) bile-salt concentration immediately after the inoculation of the probiotic bacterium into the MRS broth. During the incubation period at 37°C for 3 hours, the control MRS broth (no addition of bile-salt) demonstrated a significant increasing population of the *L. acidophilus*, while the MRS broths with 1 and 2% (w/v) bile-salt concentrations produced slightly decreasing populations of *L. acidophilus* in the middle of the incubation period followed by increasing the bacterium population at the end of the incubation period. This result strongly indicated that the *L. acidophilus* strain applied in this research was able to resist the bile-salt condition after some adaptation to the hostile environment.

When the mung bean milk samples were kept for 15 days at 4°C, the number of *L. acidophilus* in the bean milk samples was significantly increased, which could be seen from the control MRS broth (no addition of bile-salt) (Figure 4.17b and 4.17c) and was consistent with the results in the previous section of 4.4.1. Contradicted with the finding in the simulated high-acid gastric condition, the higher population number of *L. acidophilus* was not significantly affected by the presence of 1 and 2% (w/v) bile-salt concentrations in the MRS broth immediately after inoculation of the bacterium.

Table 4.16 The survival slopes of *L. acidophilus* in bile-salt conditions during the shelf-life of the *L. acidophilus* in mung bean milk at 4°C for 15 days

Storage period of *L. acidophilus* in mung bean milk (days)	bile-salt concentration in MRS broth (%)(w/v)	The survival slopes of *L. acidophilus*
0	0	0.26 ± 0.02^{d}
	1	0.07 ± 0.04^{b}
	2	0.17 ± 0.01^{c}
7	0	0.15 ± 0.01^{c}
	1	0.03 ± 0.01^{a}
	2	0.01 ± 0.02^{a}
14	0	0.25 ± 0.00^{d}
	1	0.05 ± 0.04^{ab}
	2	0.05 ± 0.02^{ab}

Values are means of three determination (n=3) with the standard deviation (±SD).

Common letters within a column are not significantly difference.

However during the incubation time at 37°C, some adaptation period by the *L. acidophilus* to the adverse environment was displayed and was similar to the finding at the beginning of the mung bean milk storage period. At the same time, there was not any adaptation time was noticed when the *L. acidophilus* was inoculated in the control MRS broth (no addition of bile-salt). Results in this section clearly demonstrated that the *L. acidophilus* strain studied in this research could survive in the bile-salt environment and the increasing population of the bacterium during the storage period of the mung bean milk did not significantly affect this resistant characteristic.