CHAPTER 4

SURVIVAL OF *B. licheniformis* IN NISIN ADDED PASTEURIZED IMITATED MILK SYSTEM AS AFFECTED BY FAT LEVELS AND STORAGE TEMPERATURES

4.1 Introduction

This section was used to understand about the effect of different fat levels on the activity of nisin to inhibit *B. licheniformis* in imitated milk system (IMS) during storage at 4 and 10ºC. The IMS was prepared from the main milk components of milk fat, lactose, casein, whey protein isolate and distilled water at different levels to simulate the composition of the normal cow milk. Fat or lipids in milk, called as milk fat, have an important function as a source of energy and an essential free fatty acid. The properties of fat will affect flavor, mouth feel and viscosity of milk and milk products (Fox and McSweeney, 1998). The milk fat consists of triglycerides, di- and mono-glycerides, fatty acids, sterols, carotenoids, vitamins and trace elements. The milk fat is present as small globules or droplets, which surrounded by a surface layer or membrane. The function of the membrane is to prevent the globules from coalescence and from the activity of lipase (Varnam and Sutherland, 1994; Bylund, 1995; Walstra *et al.*, 1999). The presence of milk fat in milk can affect the activity of nisin, since nisin has a hydrophobic characteristic. There is a possibility that the nisin molecule is conjugated with lipid components in food causing nisin to be unavailable as an antimicrobial agent (Thomas *et al.*, 2000). It had been reported that milk fat reduced the nisin activity (Jung *et al.*, 1992). The report showed that the activity of nisin would be reduced to approximately 33% in skim milk and more than 88% in milk with 12.9% fat content. In contrast, another report showed no significant difference in the activity of nisin to inhibit *Listeria innocua* in whole and skim milks (Zapico *et al.*, 1999).

This chapter of study was focused in understanding the effect of fat on the activity of nisin to inhibit *B. licheniformis* isolated from pasteurized milk in IMS. The result from this study would be used to optimize the application of nisin in inhibiting

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Bacillus spp. in pasteurized milk and improving the microbial quality of the milk products.

4.2 Materials and methods

4.2.1 Preparation of imitated milks system (IMS)

An IMS solution was prepared using Ultra-High-Temperature whipped cream (Anchor®, New Zealand), lactose solution (Fonterra, New Zealand), casein solution (BBA, France), whey protein isolate solution (Arla, Denmark) and distilled water. The lactose and whey protein isolate solutions were sterilized by 0.45µm membrane filter syringes (Acrodisc®, USA), while microorganisms in the casein solution and distilled water were removed by heating the liquid at 121ºC for 15 min. The final IMS solution contained 4.02±0.02% (w/v) fat, 3.32±0.03% (w/v) protein and 4.48±0.03% (w/v) lactose representing the normal composition of cow's milk that had 4% fat, 3.24% protein and 4.6% lactose (Harding, 1999).

4.2.2 *B. licheniformis* culture preparation

B. licheniformis used in this section was isolated from the pasteurized milk, purified by transferring the isolated bacteria 2 times on Plate Count Agar (PCA) (Merck, Germany), Gram staining following a procedure of Harrigan (1998) and identified the cell structure using a microscope (Olympus, USA). The isolated culture was subjected to some biochemical reactions, including Hugh and Leifson's, starch hydrolysis, acid from mannitol, citrate utilization and Voges-Proskauer (Holt *et al*., 1994; Priest, 1989; Harrigan, 1998) and identified its species using an API 50 CHB test kit (BioMerioux®, France). The result of the API test showed that one of the isolated colonies was *B. licheniformis* with an identification percentage of 92.4%. To produce spore, the *B. licheniformis* culture was grown on Nutrient Agar (NA) (Oxoid, England) for 1 week at 30ºC. After checking that the sporulation occurred for more than 90% of the culture by conducting spore staining using malachite green solution (Harrigan, 1998), the bacilli spore was harvested using sterile distilled water. The spore suspension was washed two times with 10 ml sterilized water and centrifuged at 4,000 x g for 10 min at room temperature before being resuspended in 10 ml sterile distilled water (Mansour *et al*., 1999; Igura *et al*., 2003). The spore suspension was

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stored at -20ºC and heated at 80ºC for 10 min to kill vegetative cells before being used in any experiment (Harrigan, 1998).

4.2.3 The effect of fat on the effectiveness of nisin to inhibit *B. licheniformis* in the IMS solutions

To study the effect of milk fat on the activity of nisin against *B. licheniformis*, concentrations of milk fat of 0, 2, 4, 10 and 18% (w/v) were incorporated into IMS solutions. These milk fat concentrations represented the fat contents of skimmed milk, semi-skimmed milk, whole milk, coffee cream and single cream, respectively (Bylund, 1995). The IMS solutions with different fat contents were prepared according to the method in the section 4.2.1. The control IMS solution in this section was the IMS solution without any fat addition. After mixing thoroughly, 100 IU/ml nisin (Nisaplin®) (Aplin & Barrett Ltd., England) and 3.87 log cfu/ml *B. licheniformis* spores were aseptically added into each of the IMS solutions. The IMS solutions were then pasteurized at 72ºC for 15 s followed by an immediate cooling in a running cold water and storage at 4 and 10ºC for 21 days. During the storage period, milk samples were separated and analyzed every 3 to 4 days interval. All the treatments were conducted in triplicate.

4.2.4 Chemical analysis.

The IMS solution containing different fat levels were analyzed for their main chemical compositions using Milkoscan 133B (Denmark). During the storage period, representatives samples of IMS solutions were regularly monitored for their total acidities and pH values. The total acidity was determined using 0.1 N NaOH (Merck, Germany) and phenolphthalein indicator (Merck, Germany) according to an American Public Health Association (APHA) method (Marshall, 1992). The result of the total acidity was expressed as % lactic acid. For the pH measurement, a pH meter (Consort C830 CE, Belgium) was employed.

4.2.5 Microbiological analysis

For the microbiological properties, samples of the IMS solutions were subjected into measurement of Total Viable Microorganism (TVM), spore and

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thermoduric bacteria counts (Marshall, 1992; Harrigan, 1998). In brief, the IMS samples were serially diluted using Maximum Recovery Dilution (MRD, Oxoid, England) before being pour-plated with Plate Count Agar (PCA) (Merck, Germany) and incubated aerobically at 30ºC for 48 h. For the spore counts, the IMS samples were heated at 80ºC for 10 min (Harrigan, 1998) prior to the enumeration process. The PCA medium and an aerobic incubation at 30ºC for 72 h were employed to determine the number of spores in the IMS solution. The thermoduric bacteria in the IMS solutions were assessed using a similar procedure as the spore count, but used a heating condition at 63.5±0.5ºC for 35 min and an aerobic incubation at 30ºC for 48 h.

4.2.6 Nisin assay

The amount of nisin in the IMS samples was regularly monitored using a method that previously described in the section 3.2.6

4.2.7 Statistical analysis

Collected data was analyzed statistically using a Factorial Experiment in Completely Randomized Design using 2 factors. The first factor was fat levels, including 0, 2, 4, 10 and 18% (w/v). The second factor was storage temperatures, which were 4 and 10°C. Then, if F value was significant ($p<0.05$), DMRT was used to determine differences between treatment means by using a SPSS program (SPSS version 10.0) (SPSS Inc., Chicago, USA).

4.3 Results and discussion

4.3.1 Chemical composition of IMS

Different fat levels of the IMS solutions were significantly affected total solid contents of the solutions (Figure 4.1). An increase in the total solids was positively corresponded with higher levels of the milk fat.

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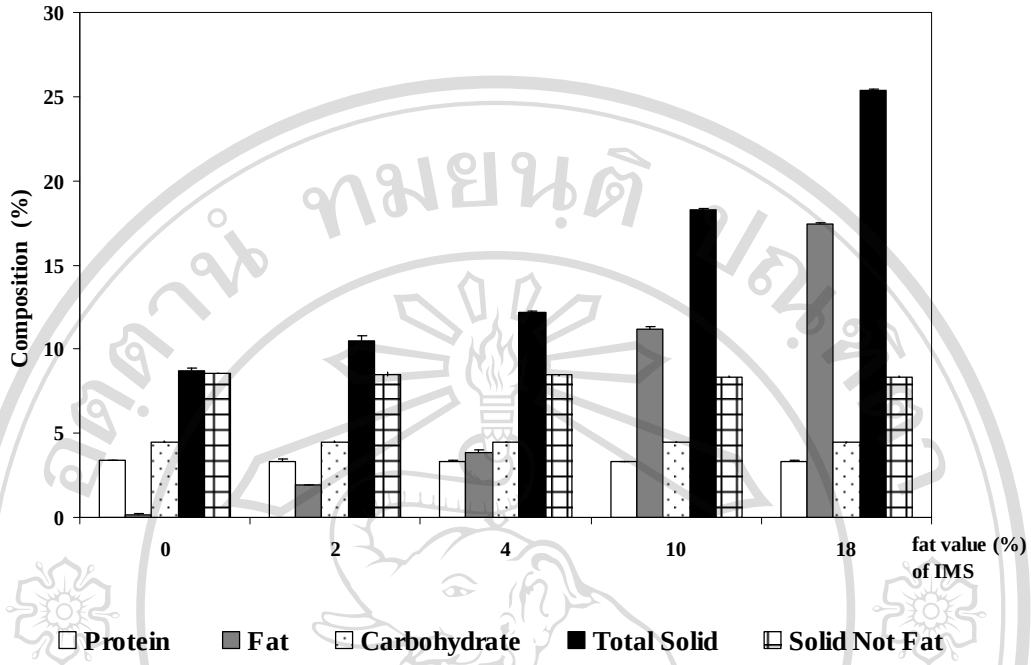

Figure 4.1 Chemical composition of IMS with different fat levels

4.3.2 The effect of fat levels on the effectiveness of nisin against *B. licheniformis* in the IMS solution

4.3.2.1 TVM count

The presence of different fat levels in the IMS solutions were found to significantly affect the TVM of the IMS directly after pasteurization and during the storage period (Figure 4.2). Using an initial *B. licheniformis* population of 3.87 log cfu/ml, the pasteurization and 100 IU/ml nisin reduced the microbial population for up to 1.51 log cfu/ml in the presence of 2% fat, which was almost similar to the control (no fat) that had a reduction of 1.45 log cfu/ml after the pasteurization process. At higher fat levels, higher reduction in the TVM of *B. licheniformis* was observed. In the presence of 18% fat, a reduction in the bacilli population for up to 2.81 log cycle was recorded.

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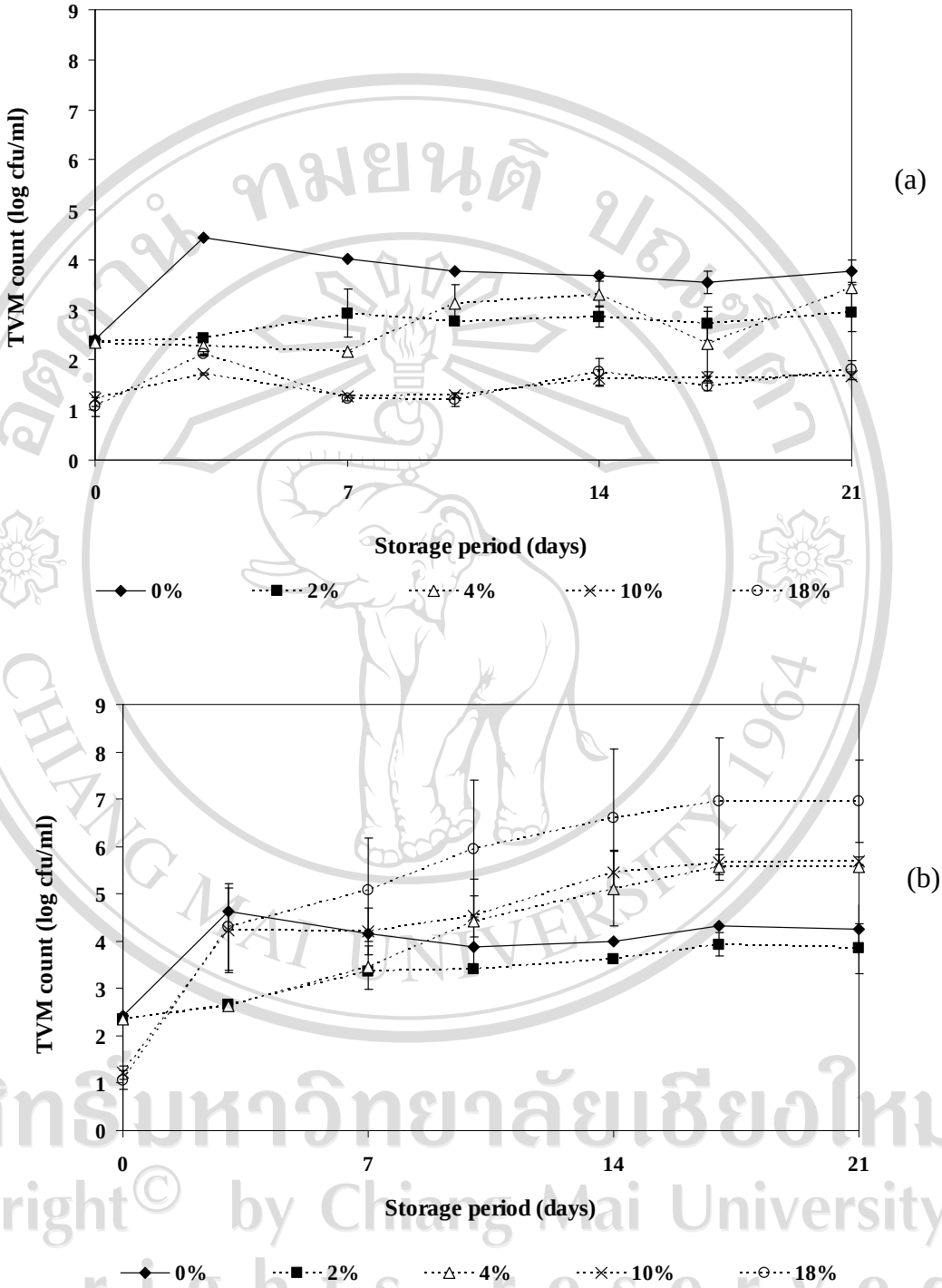

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Figure 4.2 Total Viable Microorganisms of IMS solutions with different fat levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).

During storage, the activity of nisin against *B. licheniformis* in the IMS solutions was affected by storage temperatures and fat levels. At 10ºC storage temperature, the control IMS had a significant increase in its microbial population to be 4.62±0.01 log cfu/ml within the first 3 days of storage. This microbial population was maintained until 21 days of storage, which could be due to the presence of nisin (Figure 4.7b). When higher fat levels of 10 and 18% were added into the IMS solutions, the TVM of the solutions were also increased significantly within the first 3 days of storage, diminishing the advantage of lower initial TVM counts. In contrast to the control, the TVM of the IMS with high fat levels was continued to be increased during the storage period, reaching a bacterial population of 6.95±0.86 log cfu/ml in the IMS with 18% fat level after 21 days of storage (Figure 4.2b). It was only the IMS with 2% fat that had significantly lower TVM numbers than the control for 17 days of storage at 10ºC. The result of the TVM enumeration showed that at higher storage temperature, the presence of higher fat levels significantly reduced the activity of nisin to inhibit the growth of *B. licheniformis*. This finding was similar to the report of Bhatii *et al*. (2004) that found the antimicrobial activity of nisin against *Listeria monocytogenes* was reduced in milk with 2 and 3.5% fat. Another report by Jung *et al*. (1992) also displayed that the nisin activity was reduced at higher fat contents. Finding in this study might be affected by the absorption of nisin onto the milk fat globules reducing its availability to act against microorganisms. The measurement of residual nisin also showed that at 10ºC storage temperature, the availability of nisin against microorganisms reduced at a faster rate than that at 4ºC storage temperature (Figure 4.7).

A different TVM result was displayed when the IMS solutions was kept at 4ºC storage temperature (Figure 4.2a). Although the control IMS showed an increase in the *B. licheniformis* population, the IMS solutions added with different fat levels could significantly maintain lower numbers of TVM counts throughout the storage period. Interestingly, higher fat levels produced lower TVM counts. Varnam and Sutherland (1994) had reported that survival of heat-treated *B. licheniformis* could grow during storage and finding in this section demonstrated that at lower storage temperature, nisin still could be effective in inhibiting the growth of *B. licheniformis* in the presence of high fat levels. There was a possibility that higher nisin availability

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after the heat treatment and during the beginning of the storage period (Figure 4.7a) provided a better microbial control in the IMS solution.

4.3.2.2 Spore count

The measurement of spores in the IMS solutions clearly displayed a better control of nisin against spore forms compared to the vegetative forms (Thomas *et al*., 2000). At the same time, higher fat levels of the IMS solutions did not reduce the activity of nisin in inhibiting the outgrowth of *B. licheniformis* spores, especially at lower storage temperature. Figure 4.3 clearly displayed that all the fat added IMS solutions contained lower spore counts directly after the pasteurization process and throughout the storage period compared to those of the control IMS (no fat). Lower spore counts were noticed at higher fat levels. An increase in the number of the spore in the control IMS corresponded to the increase in the TVM of the solution (Figure 4.2). However, a significant increase in the TVM counts of the IMS with high fat levels stored at 10ºC was not followed by an increase in the spore forms, demonstrating the effectiveness of nisin against the later bacterial form. Since the effect of nisin could be sporostatic rather than sporocidal, an increase in the spore count could be noticed after 10 days of storage at 10ºC in the IMS solutions with high fat levels. A similar finding was reported by Mansour *et al*. (1999) that found the presence of 25 IU/ml nisin could inhibit the spores outgrowth. However, when nisin was absence, regrowth of spores could occur within 10 to 24 h.

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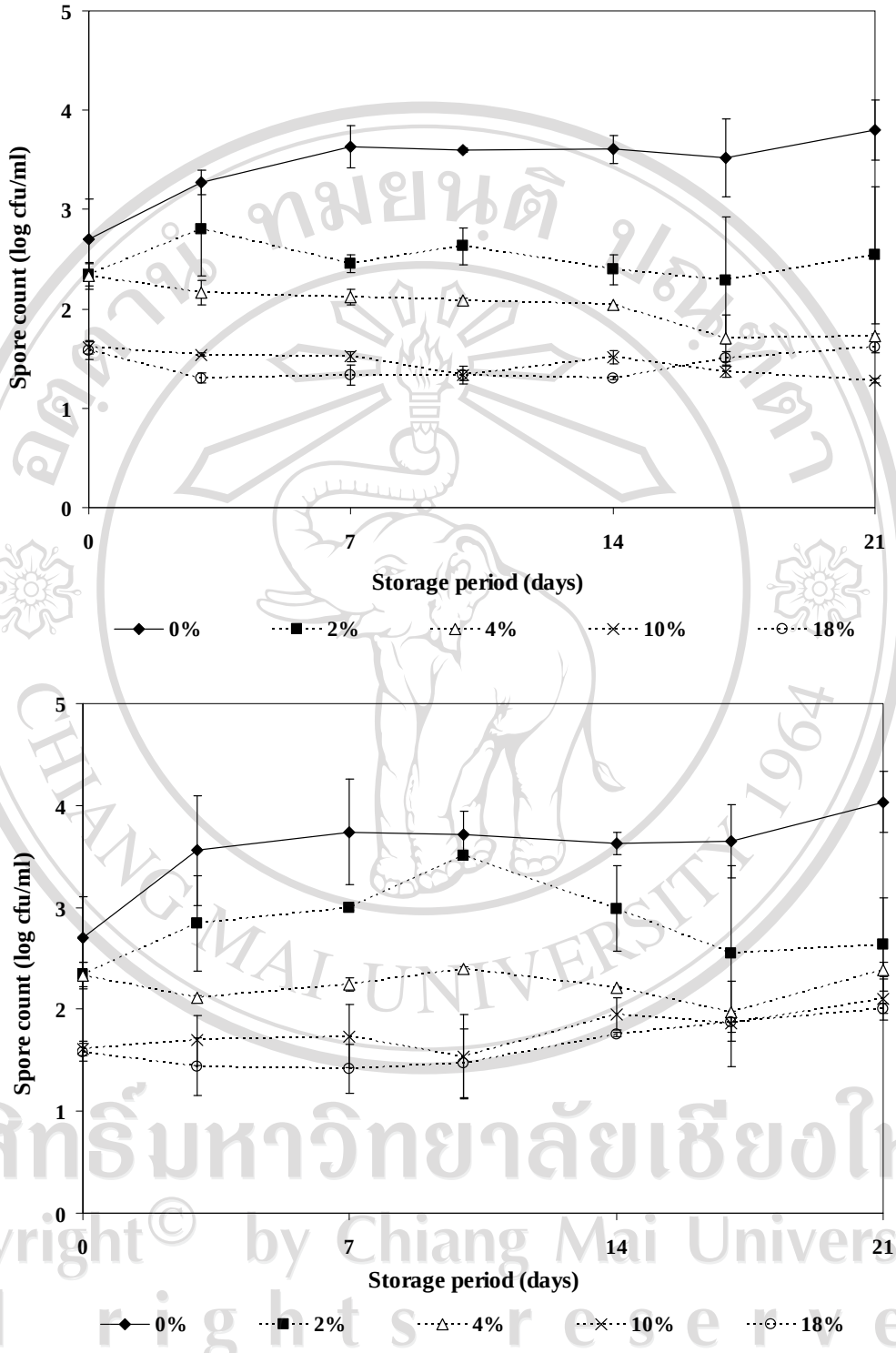

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Figure 4.3 Spore counts of IMS solutions with different fat levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).

4.3.2.3 Thermoduric count

The effectiveness of nisin against thermoduric bacteria in the IMS solutions was also affected by the storage temperatures and fat levels (Figure 4.4). At higher fat levels, significant reduction in the thermoduric counts compared to those with lower fat levels and the control was found after the pasteurization process. During the storage period, the storage temperature had a significant influence on the growth of thermoduric *B. licheniformis* in the presence of nisin. At 10ºC storage temperature, a steady increase in the thermoduric count was observed in the IMS with high fat levels reaching microbial counts that were not significantly different than that of the control IMS at the end of the storage period. On the other hand, at lower storage temperature, all the fat added IMS solutions had a slow increase in their thermoduric counts and were significantly lower than the control IMS after 21 days of storage. This finding indicated that at higher fat levels and higher storage temperatures, higher levels of nisin was needed to inhibit the growth of thermoduric *B. licheniformis*.

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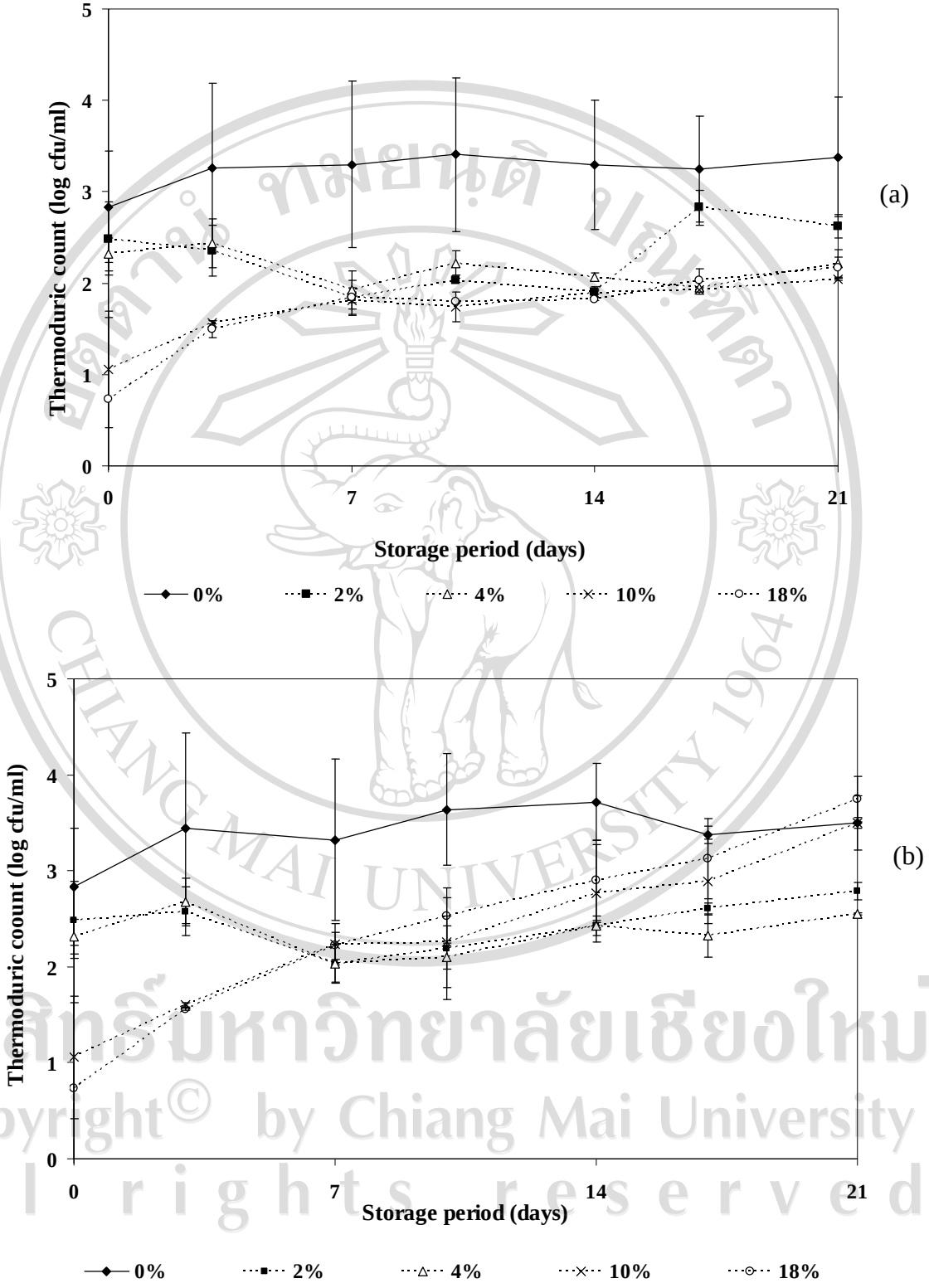

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Figure 4.4 Thermoduric counts of IMS solutions with different fat levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).

4.3.2.4 pH value

Figure 4.5 showed changing in the pH of IMS solutions during 21 days storage at 4 and 10ºC. At both storage temperature, all the IMS solutions experienced significant reduction in their pH values. Higher pH reduction was observed at higher storage temperature, which corresponded with higher microbial counts (Figure 4.2). This finding confirmed the growth of *B. licheniformis* in the IMS solutions and production of acid as their by products. Priest (1989) also mentioned that most strains of *Bacillus* produced enzyme proteases which hydrolyze gelatin and/or casein.

4.3.2.5 Acidity value

The acidity of the IMS solutions significantly increased during the storage period, which corresponded to the pH results. Higher increase in the acidity was also demonstrated at higher storage temperature. A similar explanation could be seen in the previous section.

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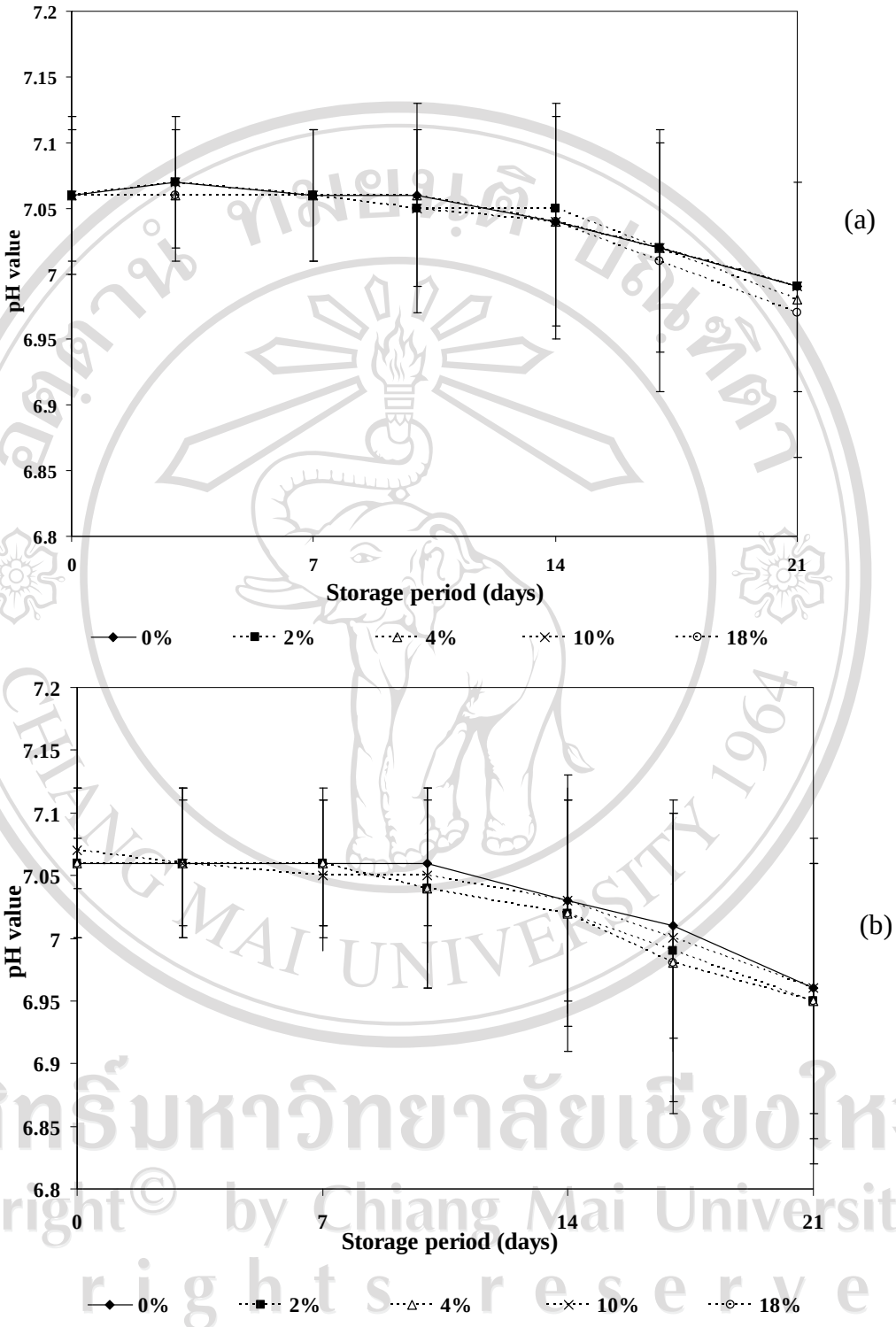

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Figure 4.5 pH values of IMS solutions with different fat levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).

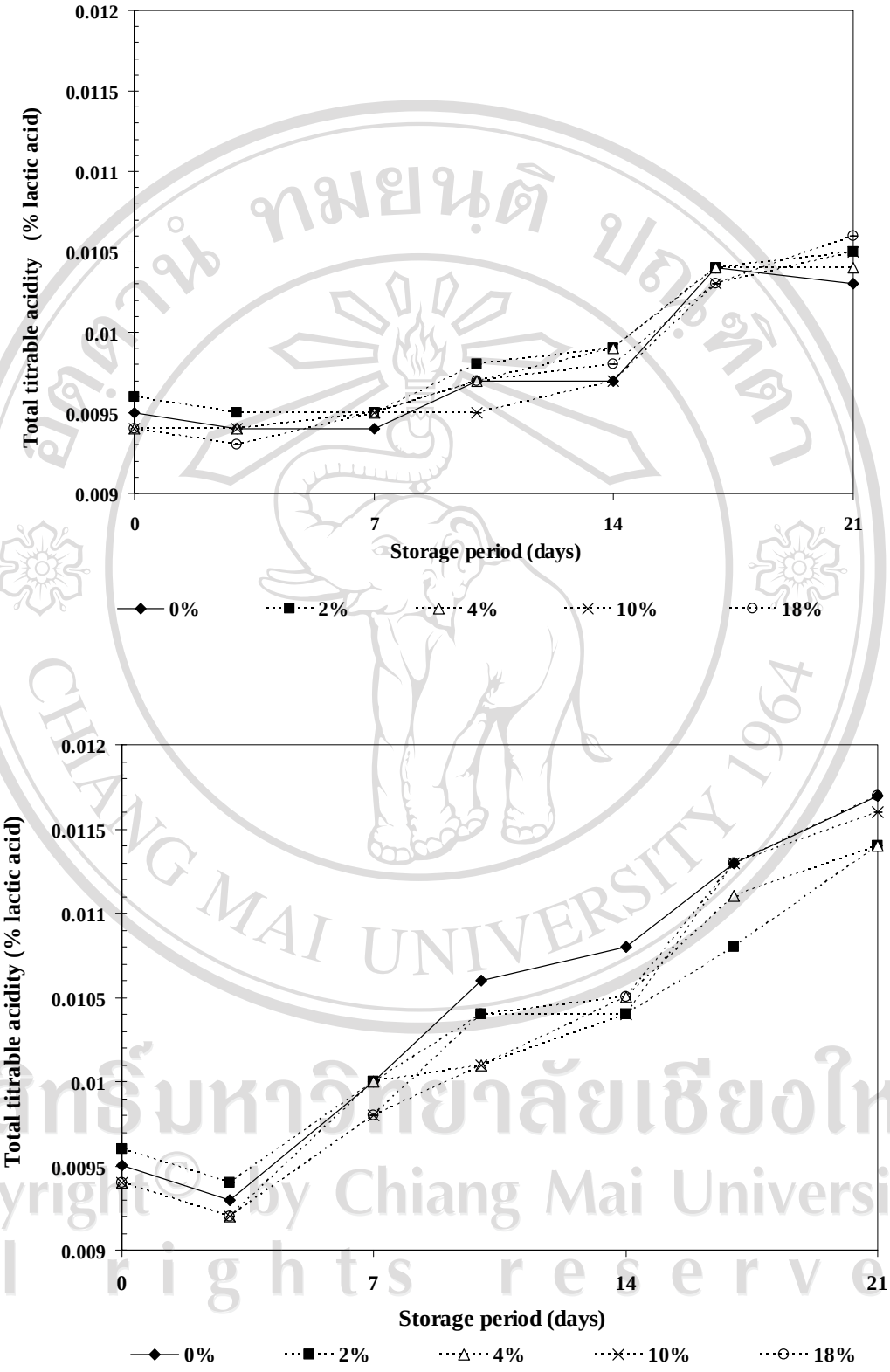

Figure 4.6 Acidity values of IMS solutions with different fat levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b)**.**

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4.3.2.6 Nisin assay

Figure 4.7 showed residual nisin activity in IMS solutions that kept at 4ºC (Figure 4.7(a)) and 10ºC (b). Reduction of the nisin activities was significantly higher at higher fat contents at both storage conditions. Longer storage time would also produce lower residual nisin concentrations. The presence of fat reduced the availability of nisin. At lower fat levels (2 and 4%), nisin activities could be detected after 21 days of storage, whereas at higher fat levels (10 and 18%), the nisin activity could not be found after 14 days of storage at both storage temperatures. The result was similar to the finding of Jung *et al*. (1992) that found skimmed milk and 12.9% fat in milk reduced the nisin activity for 33 and 88%, respectively. There was a possibility that nisin might absorb onto milk fat globules causing it to be unavailable to active against microorganisms (Jung *et al*., 1992; Zapico *et al*., 1999).

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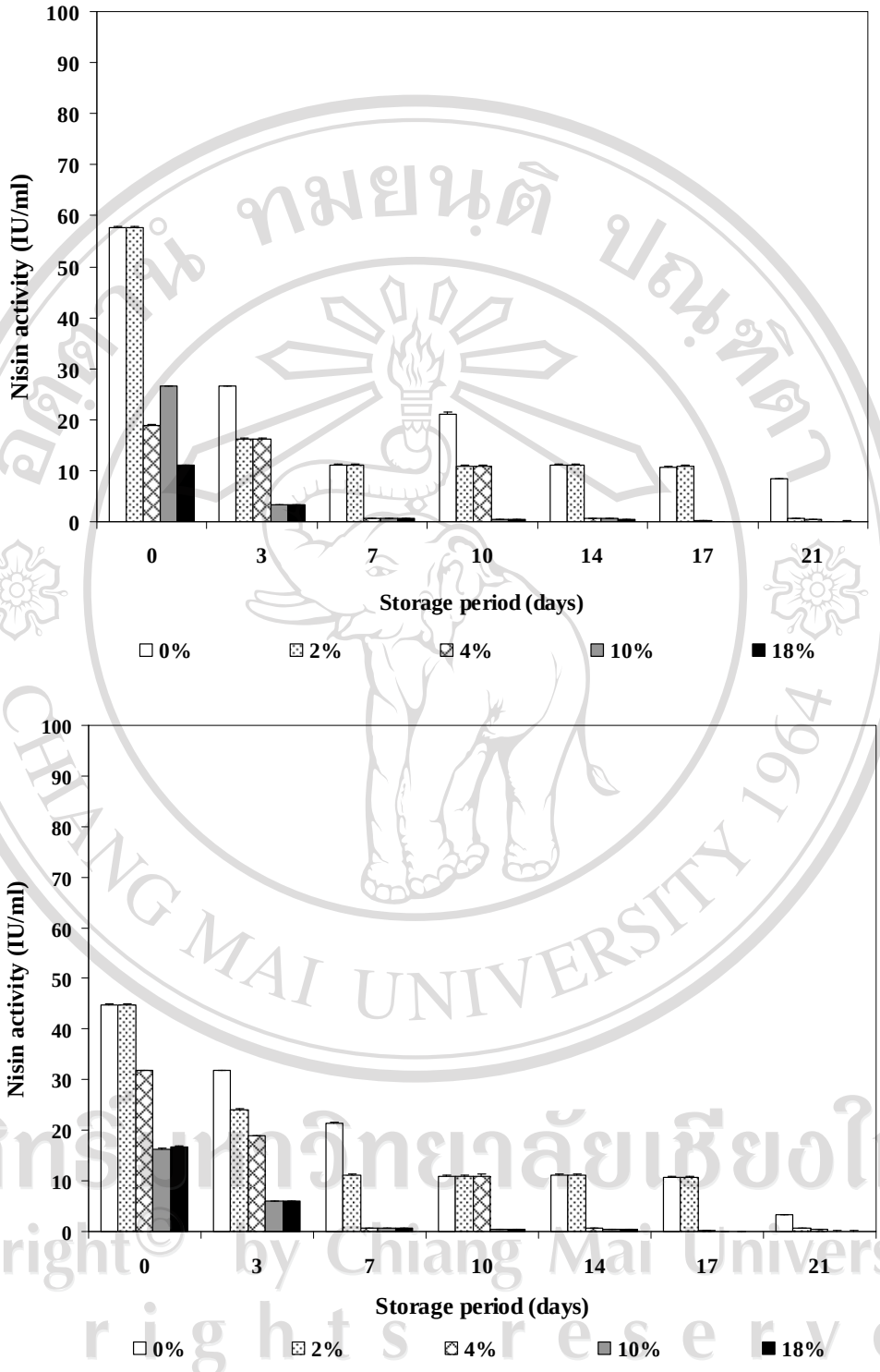

Figure 4.7 Nisin concentrations of IMS solutions with different fat levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).

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4.4 Conclusions

The fat levels were found to significantly affect the survival of *B. licheniformis* in the nisin added IMS solutions directly after a pasteurization treatment and during storage at 4 and 10°C. Higher fat contents in the IMS were significantly induced higher TVM count and significantly affected the activity of nisin to inhibit the bacilli at 10°C. At the same time, the presence of fat did not affect the effectiveness of nisin to inhibit spore germination at lower storage temperature. The thermoduric count results also indicated a lower effectiveness of nisin against vegetative cells compared to that of the spore and the presence of high milk fat would reduce more of the nisin antimicrobial activity against the target microorganism. The finding in this study supported the results of other research that the activity of nisin depended on the chemical composition in food, especially fat in dairy products. It may be useful for dairy processors to consider the concentration of nisin applied in their products based on their fat levels and storage conditions.

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