CHAPTER 6

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

6.1 Introduction

In this research, imitated milk system (IMS) was prepared from milk components to represent normal whole milk that is composed of protein, lactose, fat and water. In general, milk is made of 88.5-89.5% water and 10.5-14.5% total solids. The components of total solids are 2.5-6.0% fat, 2.9-5.0% protein, 3.6-5.5% lactose and 0.6-0.9% minerals (Porter, 1983; Walstra and Jenness, 1984; Tungjaranchai and Kavila, 1988; Bylund, 1995; Harding, 1999). About 95% of nitrogen in milk is in the form of protein. Milk proteins can be divided into two parts. The first group is casein, consists nearly 80% of the total protein in milk. Almost all casein in milk is present in casein micelles. The other group is whey protein, which is about 20% (Walstra and Jenness, 1984).

In milk and milk products, most of bacteria, particularly psychrotrophic, will cause spoilage in dairy products. The most important Gram positive psychrotropic microorganism is genera *Bacillus* (Varnam and Sutherland, 1994; Sorhaug and Stepaniak, 1997; Harding, 1999). *B. licheniformis* is a Gram positive aerobic spore forming bacterium that has been reported to be present in milk and can survive a drying process (Priest, 1989; Crielly *et al*., 1994; Wirjantoro *et al*., 2001; Chen *et al*., 2003). To prevent the growth of *B. licheniformis* in milk products, an application of antimicrobial compound is one of the alternative solutions. Nisin is an antimicrobial substance that is produced by *Lc. lactis* subsp. *lactis* (Thomas *et al*., 2000) and has been widely used as a preservative in processed cheeses and cheese spreads (Delves-Broughton, 1990).

The stability of nisin depends on its solubility, pH of the solution, chemical composition of the solution and temperature (Thomas *et al*., 2000). For instance, nisin has been reported to be reversibly adsorbed by some proteins. This adsorption probably accounted for protective effect when nisin solutions were exposed to heat

(Hall, 1966). Due to these reasons, some researches have been conducted to study the role of food components on the activity of nisin to inhibit spoilage and pathogenic microorganisms. In this study, a particular attention was given to milk protein. The study aimed to investigate the role of casein and whey protein isolate (WPI) on the effectiveness of nisin to inhibit *B. licheniformis* in IMS solutions during storage at 4 and 10°C.

6.2 Materials and methods

6.2.1 Preparation of imitated milks system (IMS)

IMS solutions were prepared according to the method in the section 4.2.1.

6.2.2 *B. licheniformis* culture preparation

The isolation and purification methods for *B. licheniformis* were described in the section 4.2.2. The *B. licheniformis* culture was kept as a stock culture on Plate Count Agar (PCA) (Merck, Germany) at -18ºC. The stock cultures of the bacilli were refreshed every 2 weeks to maintain the viability of organism. For the production of the bacilli spores, a similar procedure as in the section 4.2.2 was followed.

6.2.3 The effect of milk protein on the effectiveness of nisin to inhibit *B. licheniformis* in IMS

The experimental procedures in this section were divided into 2 sub-sections that represented 2 milk proteins of casein and whey protein. To understand the role of casein, IMS solutions were prepared from 4% (w/v) fat, 4.7% (w/v) lactose, 0.64% (w/v) WPI and different casein concentrations, which were either 0, 2.5, 5.0, 7.5 or 12.0% (w/v) (Figure 6.1). The IMS solution without any casein supplementation was used as a control treatment in the casein subsection experiment. For the effect of WPI on the activity of nisin against *B. licheniformis*, the basic IMS solutions were made from 4% (w/v) fat, 4.7% (w/v) lactose and 2.6% (w/v) casein (Figure 6.7). For the WPI levels, concentrations of 0, 1, 2 and 4% (w/v) were investigated. For the WPI experiment, the control treatment was the IMS solution without any WPI addition. After thoroughly mixed the IMS solutions, *B. licheniformis* culture at counts between 3.70-3.77 log cfu/ml and 100 IU/ml nisin was aseptically incorporated into each of the

solutions. The addition of nisin was done 30 min before the heat treatment. The IMS solutions were then heat treated at 72ºC for 15 s, immediately cooled down in a running cold water and stored at 4 and 10ºC for 21 days. Every 3 to 4 days interval, representative samples from each treatment were analyzed for their chemical and microbiological characteristics. All the treatments were done in triplicate.

6.2.4 Microbiological analysis

6.2.4.1 Total viable microorganisms

The number of total bacteria in raw milk samples was determined by a Standard Plate Count (SPC) method (Marshall, 1992).

6.2.4.2 Thermoduric bacteria

The analysis of thermoduric bacteria was carried out by heating the milk samples in a water bath (Memmert®, Germany) at 63.5±0.5°C for 35 min. After the time finished, the samples were removed from the water bath and cooled rapidly in ice water. The milk samples were diluted as appropriately and plated on Plate Count Agar (PCA). The plates were incubated at 30°C for 2 days (Harrigan, 1998).

6.2.4.3 Spore count

The number of spores in milk samples was measured by heating the samples in a water bath at 80°C for 10 min. The samples were then cooled and plated on PCA. The plates were incubated at 30°C for 2 days to allow the growth of mesophiles microorganisms (Harrigan, 1998).

6.2.5 Chemical analysis

6.2.5.1 Total acidity measurement

Milk samples of 9 g were measured and transferred into 100 ml beakers. An amount of 0.5 ml phenopthalein was added into the milk samples. The samples were then titrated against 0.1 N sodium hydroxide until a pink color appeared for 30 s (Marshall, 1992).

6.2.5.2 pH measurement

pH values of the milk samples were measured by a pH-meter (Consort C830, Belgium).

6.2.6 Nisin assay

Nisin concentration in the milk samples was determined by an agar plate diffusion method using *Micrococcus luteus* as a test microorganism (Fowler *et al*., 1975; Pongtharangkul and Demirci, 2004).

6.2.7 Statistical analysis

Data from this section was divided into 2 based on the milk protein types. For the casein result, collected data was statistically analyzed using a 5x2 Factorial Experiment in Completely Randomized Design. The first factor was casein concentrations, including 0, 2.5, 5.0, 7.5 and 12.0% (w/v) and the second factor was storage temperatures of 4 and 10°C. If F value was significant, DMRT was used to determine differences between treatment means by using a SPSS program (SPSS version 10.0).

Results from whey protein isolate experiments were analyzed statistically using a 4x2 Factorial Experiment in Completely Randomized Design. The first factor was whey protein concentrations, which were 0, 1, 2 and 4% (w/v) and the second factor was storage temperatures of 4 and 10°C. If F values were significant, DMRT was used to determine differences between treatment means by using a SPSS program (SPSS version 10.0) (SPSS Inc., Chicago, USA).

6.3 Results and discussion

6.3.1 Chemical composition of IMS solutions with different casein levels

As with the fat and carbohydrate, increasing casein levels in the IMS solutions produced significant increases in total solid and solid not fat (SNF) contents (Figure 6.1). A higher protein level in the IMS solutions than the actual added value of the casein concentrations was attributed to the fixed supplementation of 0.64% WPI in the solution.

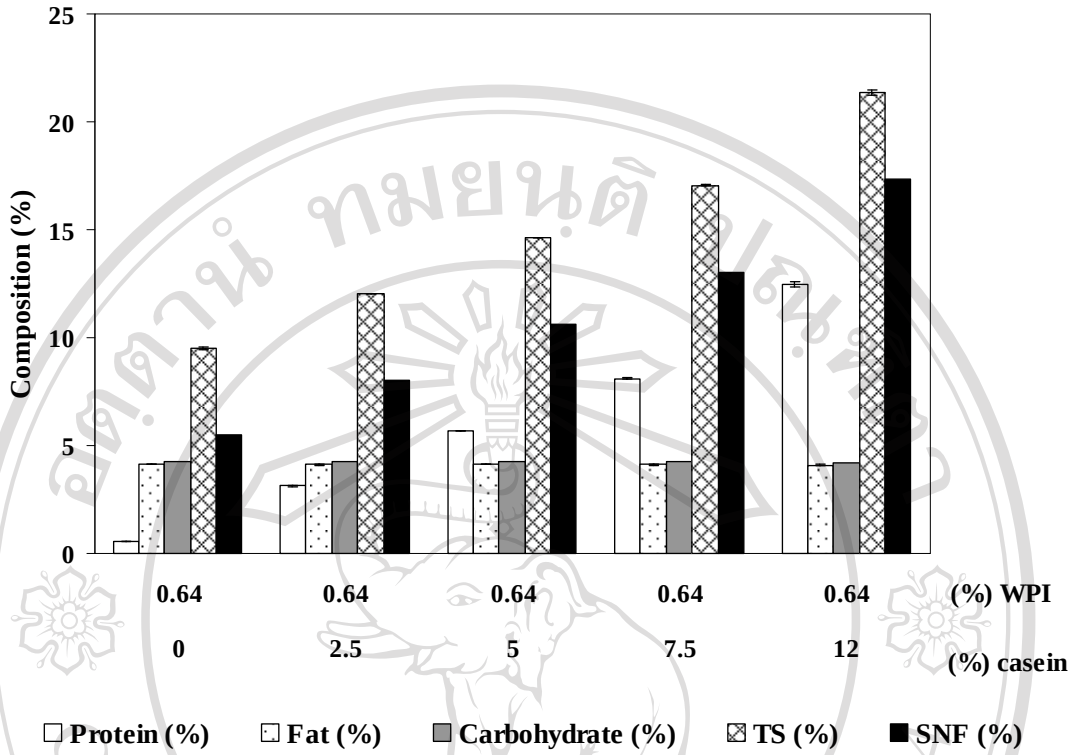

Figure 6.1 Chemical composition of IMS solutions with different casein levels.

6.3.2 The effect of casein levels on the effectiveness of nisin against *B. licheniformis* in the IMS solutions

6.3.2.1 TVM count

The result of the TVM measurement (Figure 6.2) clearly displayed that different casein levels did not significantly affect the survival of *B. licheniformis* during the pasteurization treatment and subsequent storage periods. After pasteurization, reduction in the bacilli population between 1.78 to 1.91 log cycle was recorded for all the treatments, which was almost similar to the results in the previous chapter for IMS solutions with low fat levels (Figure 4.2). During storage, storage temperature was the main factor that significantly influenced the growth of *B. licheniformis*. At 10ºC, a maximum population between 6.28 to 7.08 log cfu/ml was recorded after 10 days of storage. After this period, the viable number of the bacilli was slightly reduced. On the other hand, the TVM count at 4ºC was maintained below 2.06 log cfu/ml throughout the storage period, which could be attributed to a

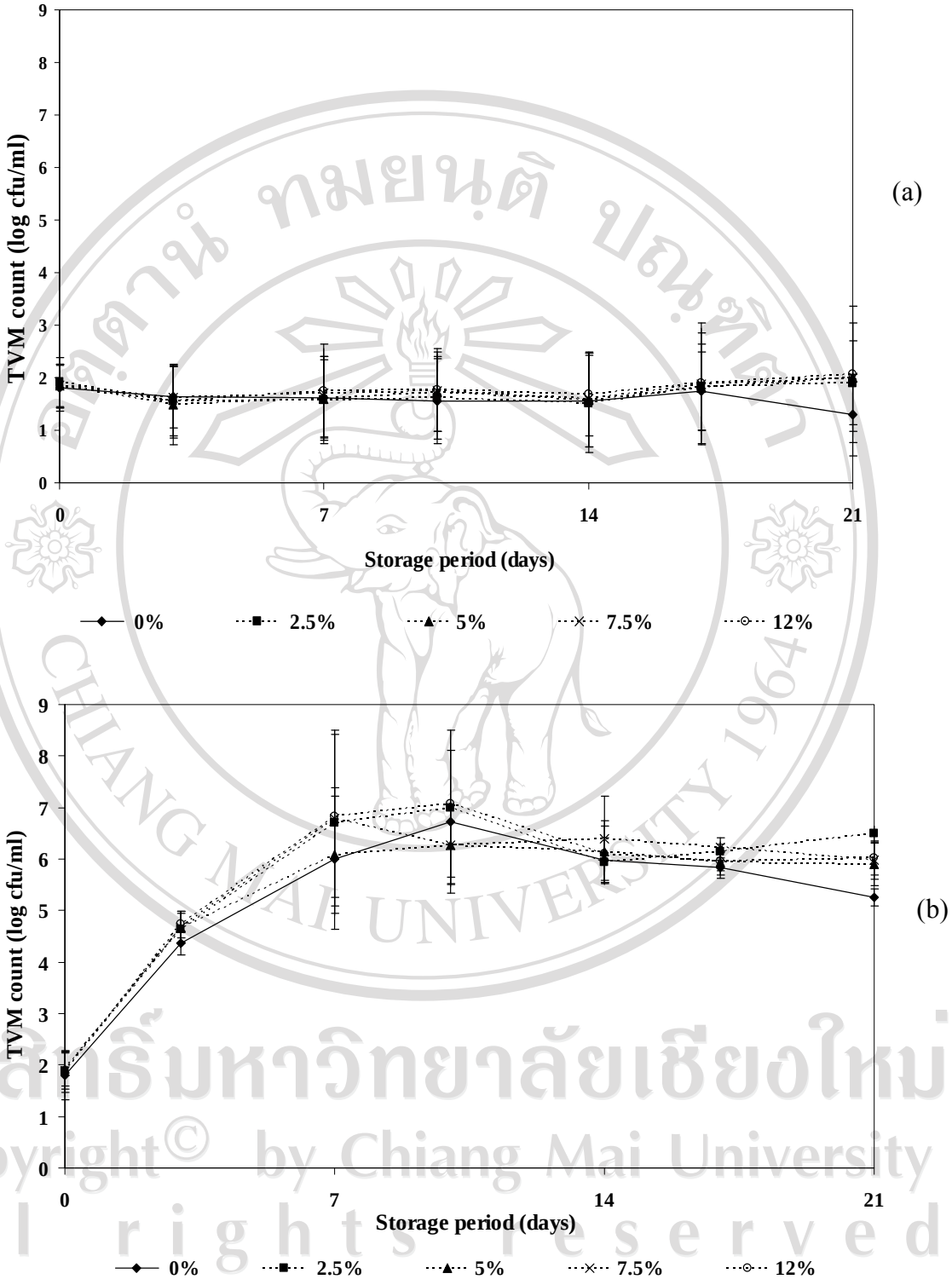

Figure 6.2 Total Viable Microorganisms of IMS solutions with different casein levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).

combination effect of nisin (Figure 6.7) and low storage temperature. Although Priest (1989) reported that most strain of *Bacillus* produced enzyme protease that hydrolyzed gelatin and/or casein, the presence of additional casein in the IMS solutions did not further increase the TVM count indicating sufficient nutrients in the IMS solutions. A slightly different finding was reported by Ganzle *et al*. (1999) who found that casein concentrations higher than 0.1 to 1.0 g/l reduced nisin activities from 0.96 to 0.60%. Thomas *et al*. (2000) suggested that nisin molecules might be bound to protein and/or the presence of protein might help in the cell recovery of microorganisms.

6.3.2.2 Spore count

Data from the spore measurement demonstrated that nisin had a better control towards the spore form (Figure 6.3) compared to the vegetative form of the bacilli (Figure 6.2). The finding confirmed the statement of Delves-Broughton (1990) that mentioned the effect of nisin against spores was more pronounced than its effect against vegetative cells of the bacteria. Different levels of casein in the IMS solutions did not produce any significant effect on the activity of nisin against *B. licheniformis* spores. Although spore counts of the IMS solutions kept at 4ºC were slightly lower than those of the samples stored at higher temperature, the effect of the storage temperature was found not to be significantly different. The highest spore numbers were generally recorded after the pasteurization process showing the effectiveness of nisin to inhibit the outgrowth of the spore form throughout the storage period. At 10ºC storage temperature, an increase in the spore count after 10 days of storage correlated with reduction in the nisin availability in the IMS (Figure 6.7) suggesting sufficient amount of nisin was needed for the whole shelf life of a product to inhibit the outgrowth of spores. The result in this section was similar to the report of Mansour *et al*. (1999) that found the presence of 25 IU/ml nisin could inhibit the spores outgrowth. The research also showed that when nisin was absence, spores could grow within 10 to 24 h at 37ºC.

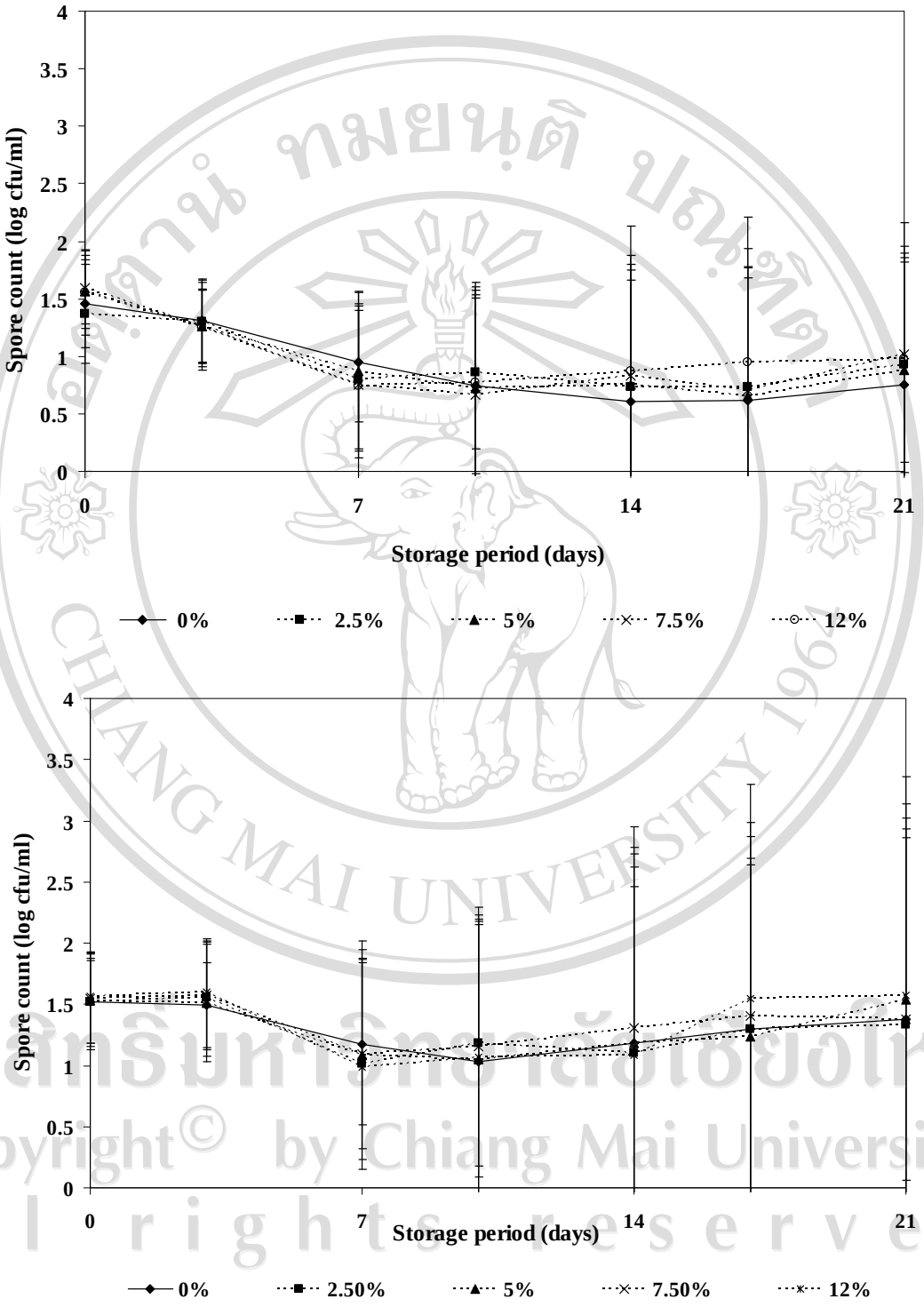

Figure 6.3 Spore counts of IMS solutions with different casein levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).

6.3.2.2 Thermoduric count

The thermoduric count results (Figure 6.4) displayed a good control of nisin towards the microorganism irrespectively to different casein levels that were present in the IMS solutions. In general, the thermoduric bacilli in the IMS solutions was between 1.50 to 1.59 log cfu/ml at the beginning of the storage period. This number was slightly reduced within the first 7 days of storage indicating the susceptibility of the heat injured microorganisms towards nisin at the beginning of the storage period before some of the thermoduric populations could have a better resistance against nisin and/or could grow in the presence of lower nisin concentrations (Figure 6.7). Between 4 and 10ºC storage temperatures, the higher storage temperature gave a better support for the thermoduric bacilli to recover. However, within 21 days of storage, the effect of storage temperature on the activity of nisin against thermoduric *B. licheniformis* was found not be significantly different.

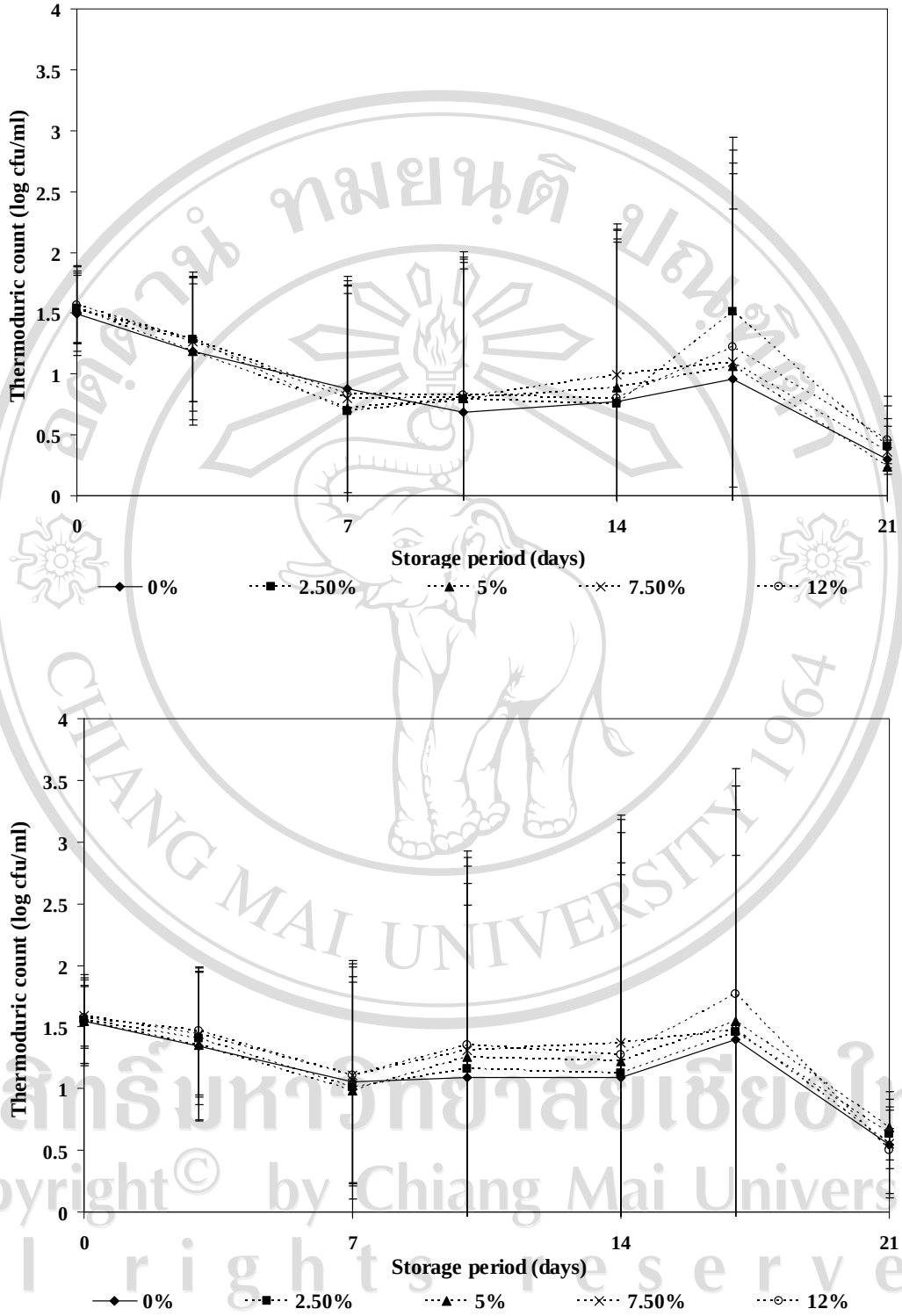

Figure 6.4 Thermoduric counts of IMS solutions with different casein levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).

6.3.3 Chemical properties of the IMS solutions with different casein levels

6.3.3.1 pH value

pH values of different IMS solutions decreased gradually during storage at 4 and 10ºC (Figure 6.5). Higher reduction in the pH of the IMS solutions kept at higher storage temperature compared to those of the samples stored at 4ºC was highly correlated with the growth of microorganisms in the milk samples (Figure 6.2). Interestingly, the pH of the IMS solutions kept at lower temperature was still reduced even though there was not any significant increase in the microbial population suggesting a continued microbial activity at this storage temperature. Different casein levels also affected the pH of the IMS solutions. Higher reduction in the pH of the samples occurred at higher casein levels. The effect could be noticed after 2 weeks storage and was significant at 10ºC storage temperature. This finding indicated that at a prolong storage period, higher casein concentrations would give a better support for microbial activity.

6.3.3.2 Acidity value

The acidity of the IMS solutions significantly increased during the storage period (Figure 6.6) that corresponded to the pH finding. Higher increase in the acidity was also demonstrated at higher storage temperature. A similar explanation could be seen in the previous section.

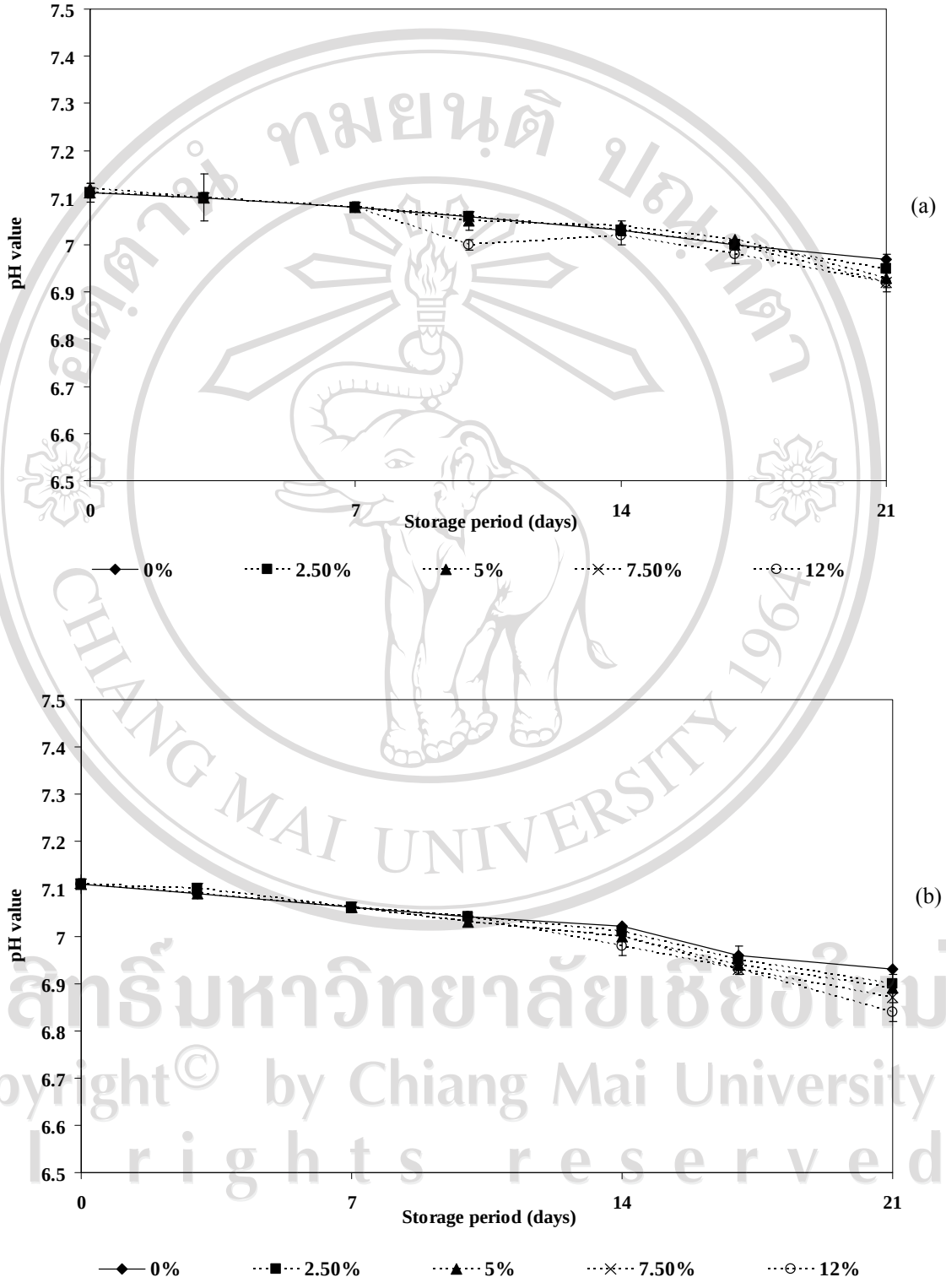

Figure 6.5 pH values of IMS solutions with different casein levels and 100 IU/ml nisin that stored at 4ºC (a) and 10ºC (b).

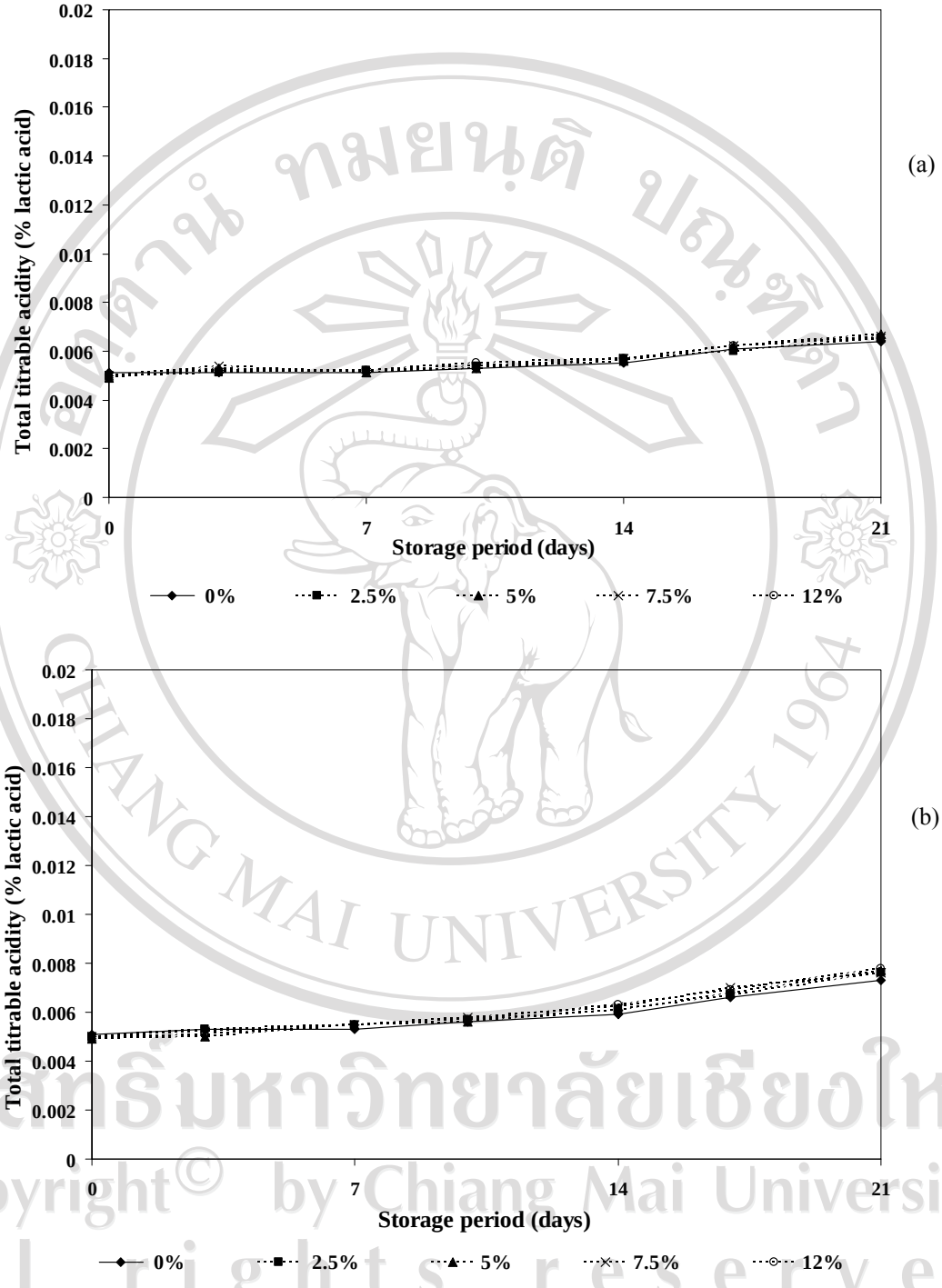

Figure 6.6 Total acidity values of IMS solutions with different casein levels and 100 IU/ml nisin that stored at 4ºC (a) and 10ºC (b).

6.3.4 Nisin assay of the IMS solution with different casein levels

Figure 6.7 displayed residual nisin activity in different IMS solutions kept at 4 and 10ºC. The presence of higher casein levels did not significantly reduce the residual nisin activities in the IMS solutions, especially when the milk stored at 10ºC. Longer storage time produced lower residual nisin activities. At 4ºC, reduction of the nisin activity was occurred at a slower rate compared to that at higher storage temperature, particularly after 10 days of storage. Nisin activities in the IMS solutions with different casein levels could be detected throughout the studied storage period.

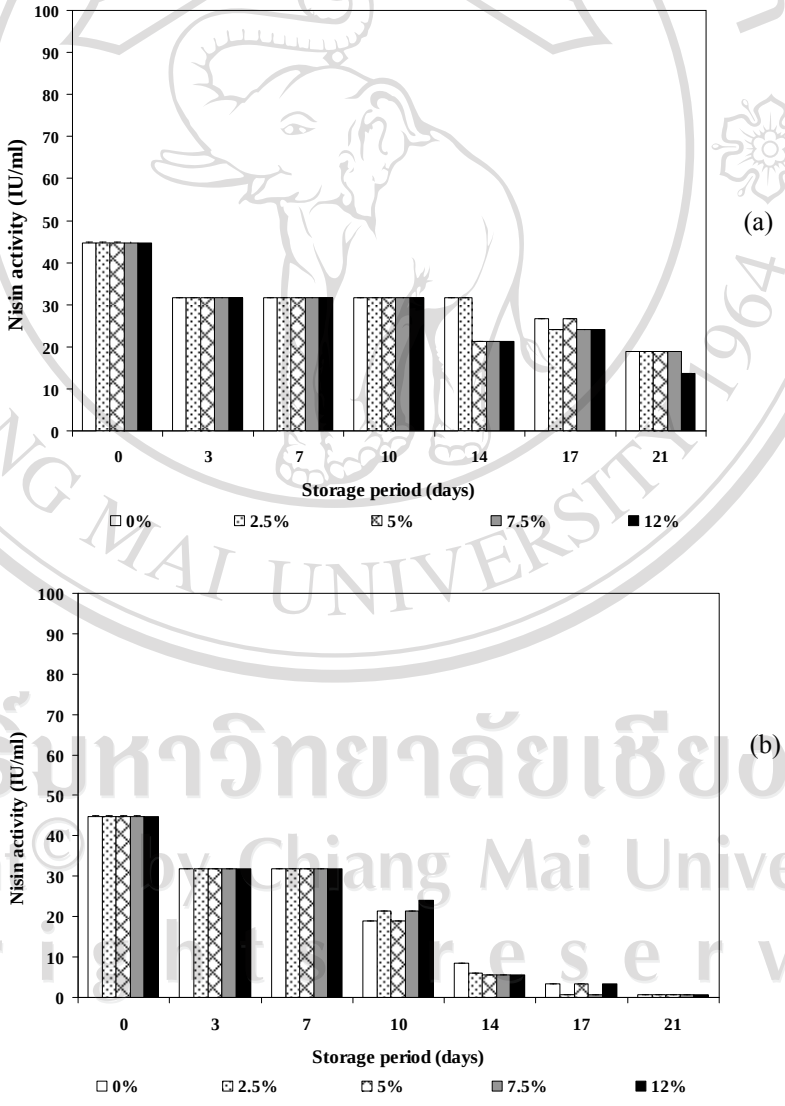

Figure 6.7 Nisin activity of IMS solutions with different casein levels and 100 IU/ml nisin that stored at 4ºC (a) and 10ºC (b).

6.3.5 Chemical composition of IMS solutions with different WPI levels

Figure 6.8 revealed that fortification of WPI in IMS solutions significantly affected the total solid and SNF contents of the solutions. The total solid and SNF contents increased with higher levels of WPI.

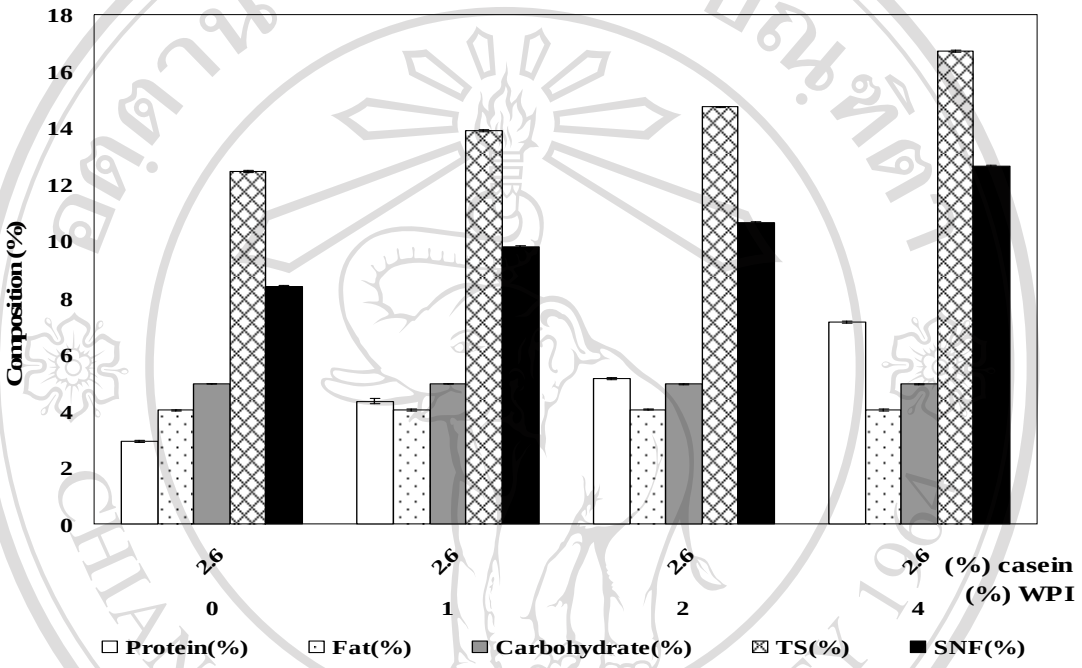

Figure 6.8 Composition of IMS solutions supplemented with different concentrations of WPI.

6.3.6 The effect of WPI levels on the effectiveness of nisin against *B. licheniformis* in the IMS solutions

6.3.6.1 TVM count

Applying a pasteurization process at 72ºC for 15 s to the nisin added IMS solutions significantly affected the vegetative cells of *B. licheniformis*. A reduction in the TVM count for up to 2.54 log cfu/ml directly after the heating process (Figure 6.9) indicated that the combination hurdle treatment had a significant effect in reducing the initial *B. licheniformis* population. The presence of nisin in the milk solution continued to affect the survival of *B. licheniformis* within the first few days of storage. In the absence of WPI (the control treatment), nisin could produce an additional

reduction of 0.31 and 0.26 log cfu/ml of the *B. licheniformis* population in the IMS solutions kept at 4 and 10ºC, respectively (Figure 6.9). This effect was reduced in the presence of WPI, especially at higher concentrations, suggesting that the nisin molecules might be bound to the protein and/or the protein might help in the cell recovery of the *B. licheniformis* population (Ganzle *et al*., 1999; Thomas *et al*., 2000).

During 21 days of storage, the effectiveness of nisin against *B. licheniformis* significantly depended on the storage temperature (Figure 6.9). In the control treatments, a significant growth of the bacilli at 10ºC storage temperature that reached a microbial population of 4.59±0.07 log cfu/ml was displayed within one week of storage followed by a steady increase in the studied microorganism population until at the end of the storage period (Figure 6.9b). On the other hand, the combination of nisin and storage temperature of 4ºC significantly and synergistically inhibited the growth of *B. licheniformis* throughout the studied storage time (Figure 6.8a). This finding was in an agreement with Montville *et al*. (1995) who reported that the effectiveness of nisin to delay bacteria growth was highly dependent on nisin concentration and temperature. Whereas a scientific report of Thomas and Wimpenny (1996) demonstrated that the combination effect of nisin, sodium chloride and temperature on *L. monocytogenes* and *Staphylococcus aureus* was affected by the target microorganism. Lowering incubation temperature and increasing sodium chloride concentrations increased the effectiveness of nisin against *S. aureus*, whereas higher temperatures and greater NaCl concentrations worked synergistically with nisin to produce a bactericidal effect against *L. monocytogenes*.

The presence of WPI in the IMS solutions significantly demonstrated an increase in the antimicrobial effect of nisin against *B. licheniformis*. Increasing the WPI levels from 1 to 4% (w/v) did not further increase the bacteriocin antimicrobial activity. This effect was clearly pronounced at 10ºC storage temperature. At 4ºC storage temperature, the effectiveness of nisin against bacilli population in the presence of different WPI levels needed to be observed at longer storage period. The effectiveness of nisin and pulsed electric fields (PEF) against *L. innocua* in whey had also been reported by Gallo *et al*. (2007). Although the report suggested that nisin

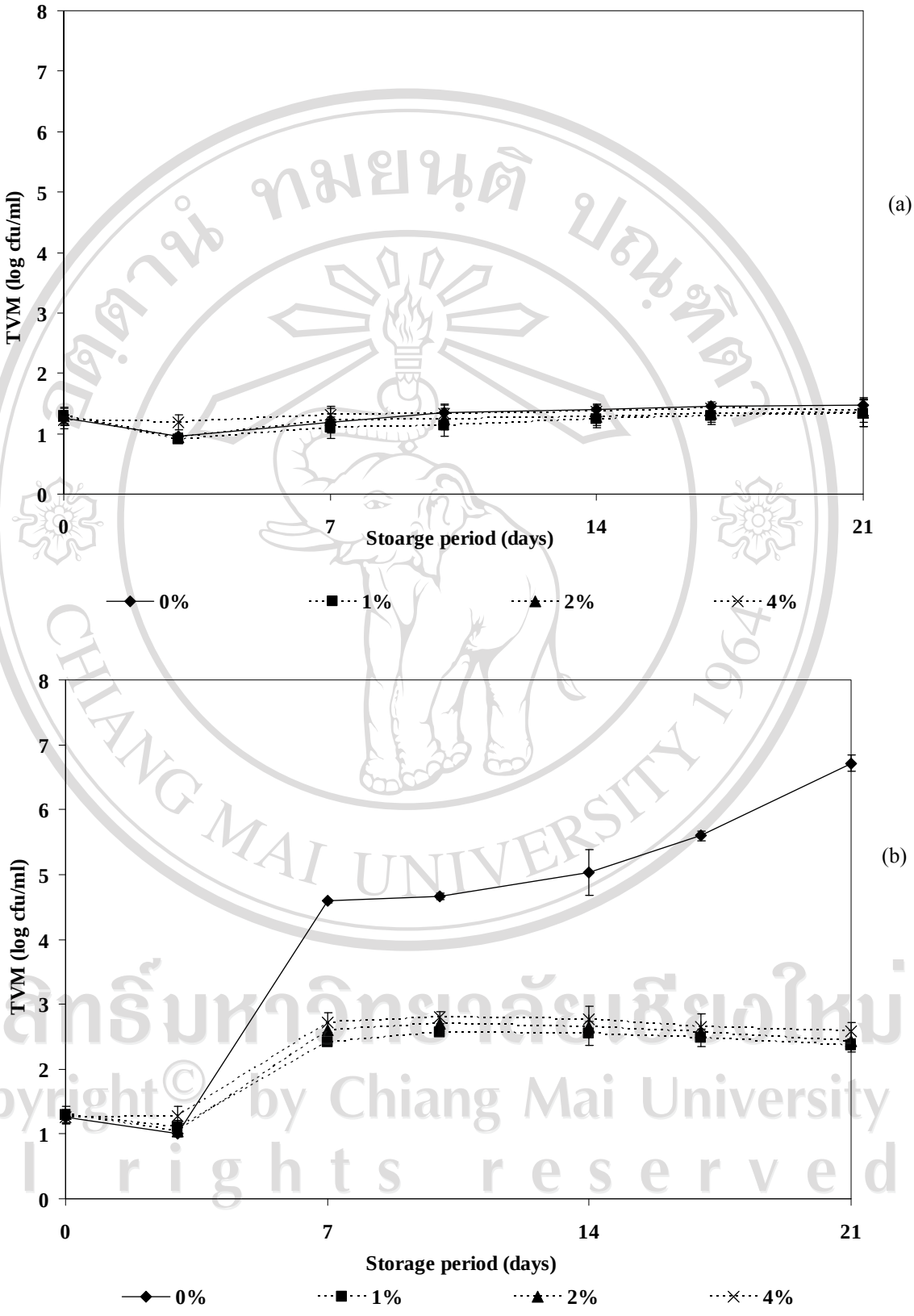

Figure 6.9 Total Viable Microorganisms of IMS solutions with different WPI levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).

could interact with whey components modified by the PEF treatment, a scientific report by Lakamraju *et al*. (1996) demonstrated that the interaction of nisin with whey protein components, such as α-lactalbumin and β-lactoglobulin, was not as strong as the bacteriocin interaction with β-casein. The last paper also showed that a weaker nisin interaction with α-lactalbumin compared to β-casein produced a higher nisin biological activity against *Pediococcus pentosaceus* FBB 61-2 after the bacteriocin contacted with the individual protein component. In this study, there was a possibility that during the recovery of the bacilli cells from heat injuries, the presence of WPI might facilitate the interaction of nisin with the bacterial cytoplasmic membrane, which was reported as the main target of nisin activity (Lins *et al*., 1999).

6.3.6.2 Spore count

In general, the number of *B. licheniformis* spores was maintained throughout 21 days storage at refrigerator temperatures (Figure 6.10). The combination of nisin and refrigerated temperature was sufficient in maintaining the number of the bacilli spores below 1.42 log cfu/ml during the studied storage period. The presence of WPI did not give any significant effect on the outgrowth of the spores. Since the nisin activity against spores was reported to be sporostatic rather than sporocidal (Montville *et al*., 1995), the presence of the bacteriocin in food throughout the shelf life period would be important.

6.3.6.3 Thermoduric count

In general, the activity of nisin against thermoduric bacteria in the IMS solutions during 21 days of storage was effective in all of the WPI levels and at both storage temperatures. The number of the thermoduric bacilli was kept below 1.37 log cfu/ml during the storage period. The effects of storage temperatures and WPI levels could be noticed after 2 weeks of storage. Higher thermoduric counts were observed at higher WPI levels or at higher storage temperature. A slow increase in the number of thermoduric bacteria after 10 days of storage at both storage temperatures was correlated with reduction of the nisin activity in the IMS solutions (Figure 6.14).

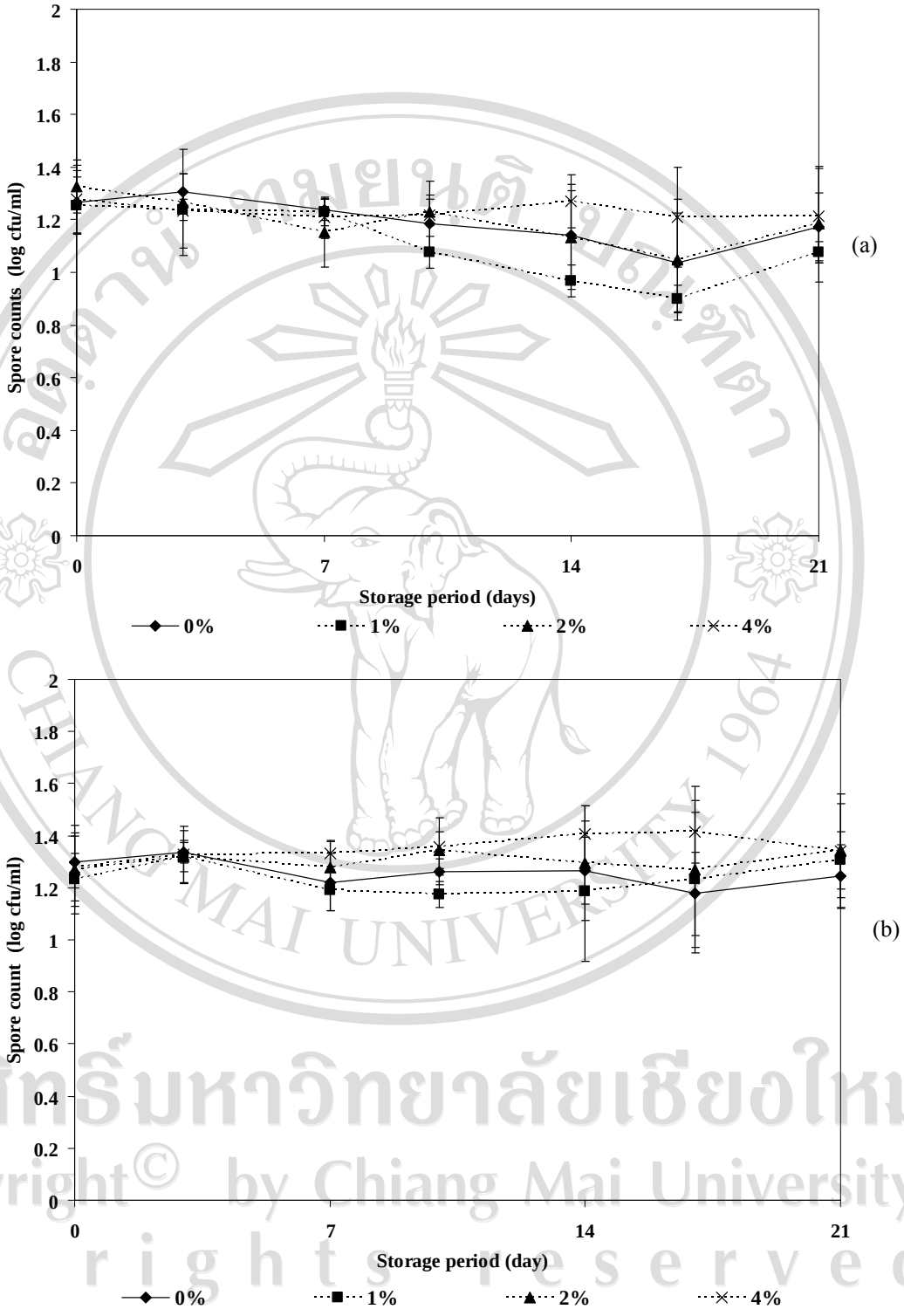

Figure 6.10 Spore counts of IMS solutions with different WPI levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).

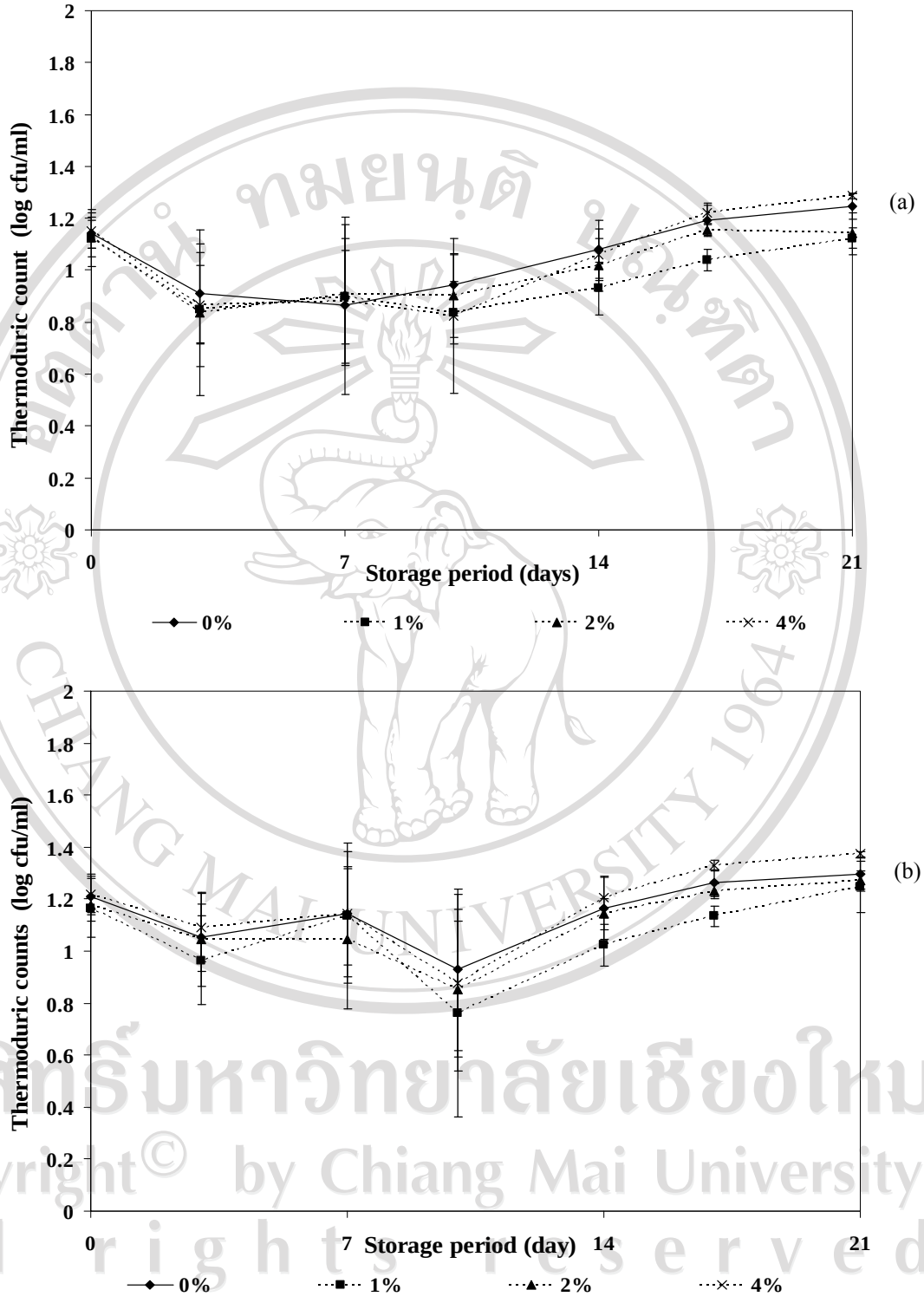

Figure 6.11 Thermoduric counts of IMS solutions with different WPI levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).

6.3.6.4. pH and acidity values

The pH of the IMS solutions was higher than the normal pH of cow milk, which was within the range of 6.6-6.7 (Walstra *et al*., 1999) (Figure 6.12). The higher pH value of the IMS could be due to the absence of mineral salts addition, such as calcium, sodium, phosphate, carbonate and citrate, that affected the ionic balance of the milk solution (Walstra *et al*., 1999). During storage at low temperatures, the pH of the milk solutions was significantly reduced together with a significant increase in the total titratable acidity (Figure 6.13). The development of milk acidity was mainly because of the growth of *B. licheniformis* that has been reported to produce acid and gas from glucose (Priest, 1989). The acidity development was higher at 10ºC storage temperature compared to those at 4ºC indicating a better growth rate of *B. licheniformis* at higher storage temperature. However, the control treatment stored at 10ºC did not produce a significant higher acidity value compared to those of the milk treatments supplemented with WPI. This finding might suggest that the presence of nisin in the milk solution affected the metabolism activity of *B. licheniformis* eventhough the microorganism itself could increase its population for up to 6.72 ± 0.13 log cfu/ml at the end of the storage period.

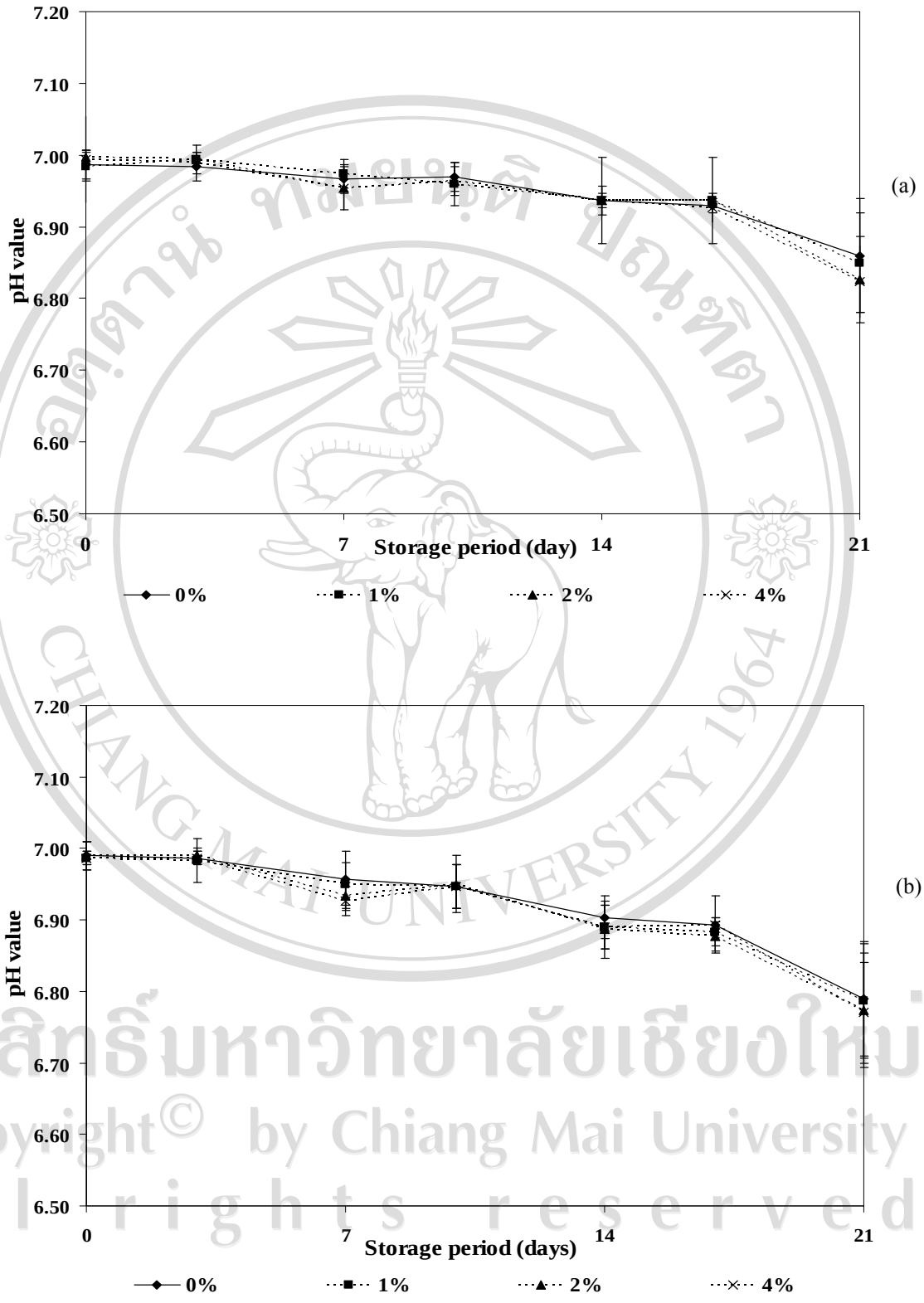

Figure 6.12 pH values of IMS solutions with different WPI levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).

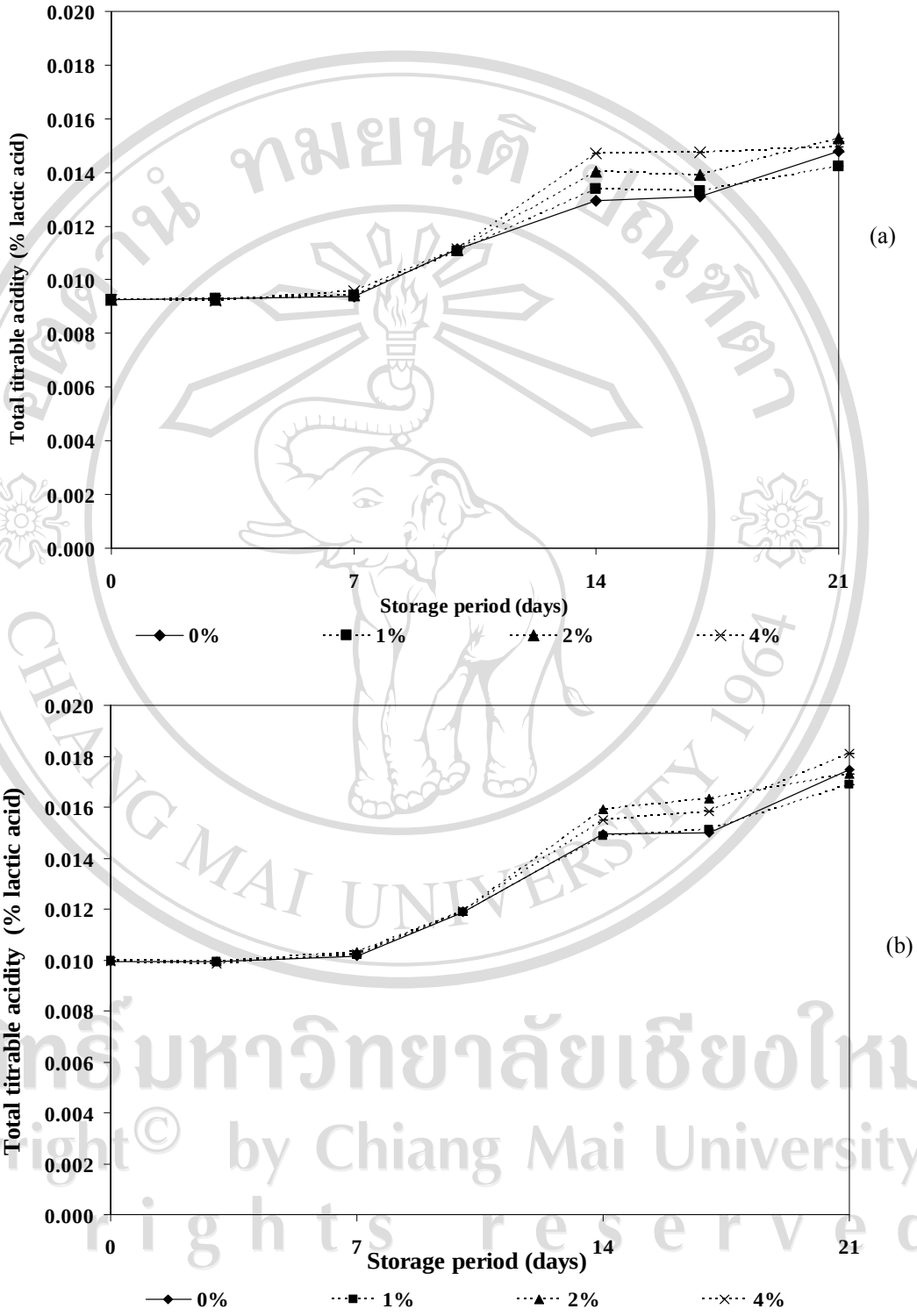

Figure 6.13 Acidity values of IMS solutions with different WPI levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).

6.3.7 Nisin assay of the IMS solutions with different WPI levels

Reduction of the nisin activities was significantly higher at higher storage temperature (Figure 6.14). Longer storage time would also produce lower residual nisin activities. At higher WPI levels, the residual nisin activities were reduced at slower rates and supported the TVM finding (Figure 6.9). After 3 days of storage, the control IMS treatment had a higher reduction rate for its nisin activities compared to those of the IMS treatments supplemented with WPI.

6.4. Conclusions

Result in this section showed that casein did not support the activity of nisin to extend the microbial quality of the IMS solutions. On the other hand, nisin could work effectively to inhibit the growth of *B. licheniformis* in the IMS solutions, particularly for its vegetative form, in the presence of different WPI levels. The effect of WPI was more pronounced at 10°C storage temperature, in which the studied microorganisms had a better growth rate. The WPI result also confirmed that the presence of fat, lactose and casein in the IMS solutions might contribute to a lower effectiveness of nisin against *B. licheniformis*, especially at higher storage temperature.

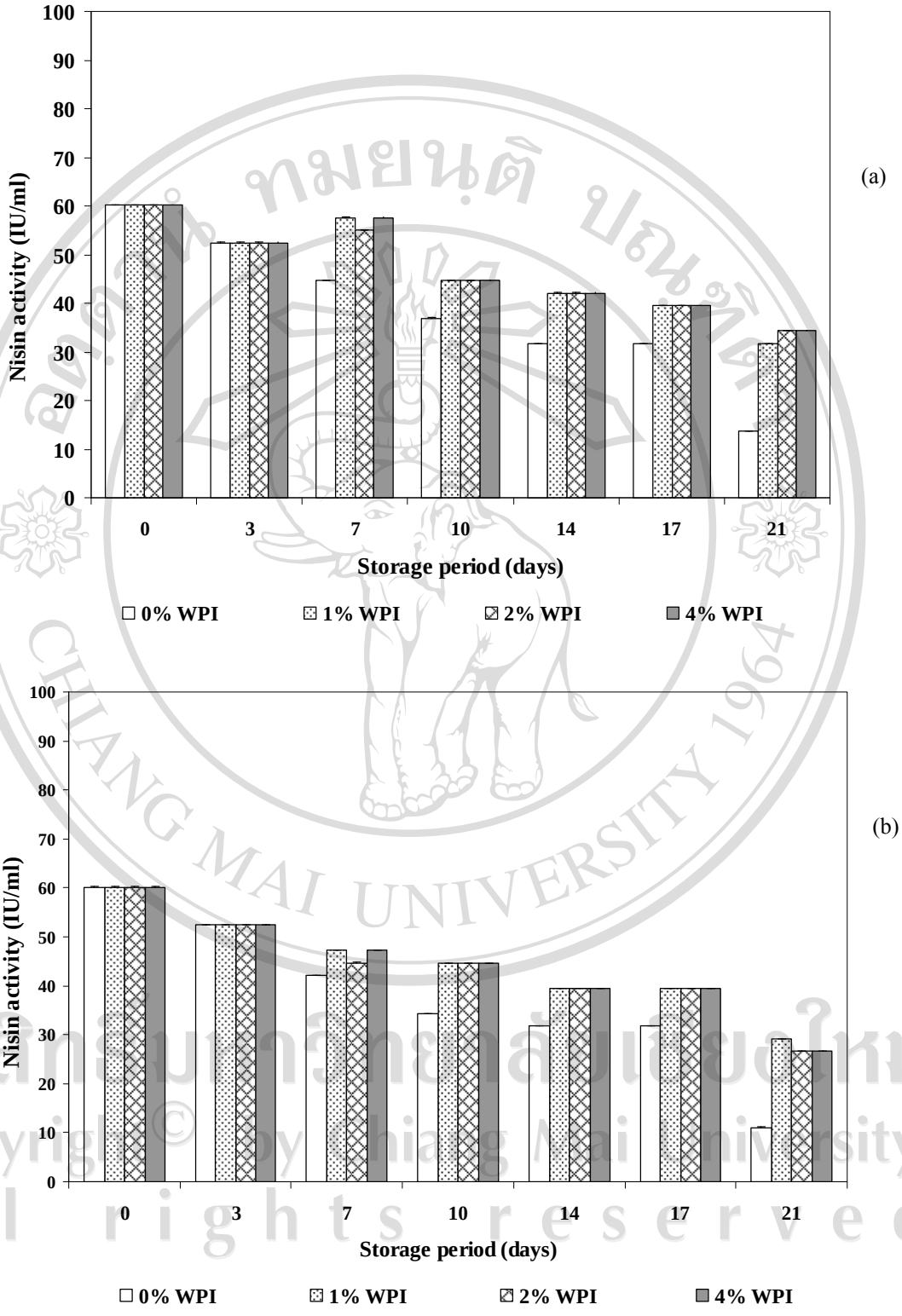

Figure 6.14 Nisin activity of IMS solutions with different WPI levels and 100 IU/ml nisin stored at 4ºC (a) and 10ºC (b).