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The combined effects of whey protein isolate concentrated and low temperature with nisin on survival of *Bacillus licheniformis* in imitated milk solution

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Combined effect of whey protein isolate and low temperature with nisin on survival of *Bacillus licheniformis* were investigated. Sterile imitated milk solution prepared from casein, milk fat, lactose and distilled water was mixed with different whey protein isolate levels of 0, 1, 2 and 4% (w/v) and 100 IU/ml nisin. After mixed thoroughly, the milk solution was aseptically inoculated with 3.74-3.78 log cfu/ml *Bacillus licheniformis*, pasteurized at 72ºC for 15 s, cooled down and subsequently stored in low temperature at 4 and 10ºC for 3 weeks. The results clearly demonstrated that *B. licheniformis* had a better growth rate at 10ºC compared to that at 4ºC even in the presence of 100 IU/ml nisin. The presence of whey protein isolate as low as 1% (w/v) could work synergistically with nisin in inhibiting the target organism. The growth of the bacilli in milk solution was accompanied with the development of milk acidity. The outgrowth of the bacilli spores could be inhibited by the presence of nisin and low storage temperatures.

Key words: pasteurized milk, whey protein isolate, nisin, *Bacillus licheniformis*, storage temperature

Introduction

Bacillus licheniformis is a Gram positive aerobic spore forming bacterium that has been reported to be present in heat-treated liquid milk and dried skim milk (Priest, 1989; Wirjantoro *et al*., 2001). Although the bacterium is not a pathogenic bacterium, the presence of the organism in milk and dairy products will cause spoilage and economical losses. Since the bacilli spores have been reported to be present in raw milk (Crielly *et al*., 1994) and the spores are heat resistant, the presence of the spores in pasteurized milk

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products can not be avoided. Moreover, one of the *Bacillus* spp., *Bacillus cereus*, had been reported to have a minimum growth temperature between 4-15ºC (Gould, 1995). Due to these reasons, inhibiting the growth of the bacilli in pasteurized milk would be desirable and might reduce the likelihood of food poisoning in the future.

To prevent the growth of *B. licheniformis*, nisin can be applied in milk products. Nisin is an antimicrobial peptide produced by *Lactococcus lactis* subsp. *lactics* (Thomas *et al*., 2000). It is ribosomally synthesized and killed closely related bacteria (Cleveland *et al*., 2001). The effectiveness of the bacteriocin against various Gram positive bacteria, including *B. cereus* (Dufrenne *et al*., 1994; Beuchat *et al*., 1997), *Bacillus coagulans* (Roberts and Hoover, 1996), *Staphylococcus aureus* and *B. cereus* (Fang *et al*., 1997), *B. licheniformis* (Bell and de Lacy, 1985), *Clostridium botulinum* (Rogers and Montville, 1994; Mazotta *et al*., 1997) and *Listeria monocytogenes* (Jung *et al*., 1992; Thomas and Winpenny, 1996; Okereke and Thomson, 1996) had been reported. The main target of nisin action is the cytoplasmic membrane of sensitive cells causing a rapid and non-specific efflux of small molecular weight compounds (Montville *et al*., 1995; Thomas *et al*., 2000). In addition, nisin has been confirmed to be Generally Recognized as Safe and has an Acceptable Dairy Intake of 2.9 mg/person/day (Thomas *et al*., 2000; Cleveland *et al*., 2001).

The effectiveness of nisin in a food system may depend on several factors, including changes in nisin solubility and charge; binding of nisin to food components (e.g. milk fat); inactivation of nisin (by proteases or food ingredients) and changes in the cell envelope of the target organisms in response to environmental factors (Gänzle *et al*., 1999; Thomas *et al*., 2000). Due to these reasons, lots of research have been conducted to investigate the role of different food components either in a model food system or in food on the activity of nisin against different spoilage and pathogenic microorganisms. In here, the effectiveness of nisin against *B. licheniformis* was evaluated using an imitated milk solution that was prepared from sterile individual milk components. The growth of the bacilli during storage at 4 and 10ºC was closely monitored to understand whether the presence of different whey protein isolate concentrations affected the action of nisin.

Materials and methods

B. licheniformis culture

B. licheniformis used in this research was isolated from the local raw 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 (Harrigan, 1998) and identified its species using an API 50 CH kit (BioMerioux®, France). The result of the API test showed that the isolated colony 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 doing spore staining using malachite green solution (Harrigan, 1998), the bacilli spore was harvested using sterile distilled water. The spore suspension was washed two times by centrifugation at 4,000 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 stored at -20ºC and heated at 80ºC for 10 min to kill vegetative cells (Harrigan, 1998; Mansour *et al*., 1999) before being used in any experiment.

Imitated milk solution

An imitated milk solution was prepared using Ultra-High-Temperature whipped cream (Anchor®, New Zealand), filtered sterile lactose solution (Fonterra, New Zealand), sterile casein solution (BBA, France), filtered sterile whey protein isolate solution (Arla, Denmark) and sterile distilled water. The final milk solution contained 4.02 ± 0.02% (w/v) fat and 4.94 ± 0.02% (w/v) lactose representing the normal composition of cow's milk that had 4% fat and 4.6% lactose (Harding, 1999). For the protein content, different whey protein isolate concentrations of 0, 1, 2 and 4% (w/v) produced milk solution with protein contents of 2.92 ± 0.03, 4.34 ± 0.09, 5.14 ± 0.03 and 7.13 ± 0.03% (w/v), respectively. After mixing thoroughly, 100 IU/ml nisin (Nisaplin®) (Aplin & Barrett Ltd., England) and 3.74 - 3.72 log cfu/ml *B. licheniformis* spores were aseptically added. The whole milk solution was pasteurized at 72ºC for 15 s followed by an immediate cooling 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.

Microbiological and chemical analysis

Milk samples were serially ten-fold diluted using Maximum Recovery Diluent (Oxoid, England) and subjected to Total Viable Microorganism (TVM) count (Marshall, 1992; Harrigan, 1998) and spore count (Harrigan, 1998). The TVM was examined using a pour-plate method and PCA (Merck, Germany) before being incubated at 30ºC for 48 h. For the spore count, milk samples was heated at 80ºC for 10 min to kill vegetable cells of *B. licheniformis* before being

diluted and followed the analysis procedure of the TVM count. The pH of milk samples was monitored by a pH-meter (Consort C830, Belgium). Whereas, the total titratable acidity of the milk was checked by titrating 10 ml of the milk samples with 0.1 N NaOH (Merck, Germany) using a phenolphthalein indicator (Merck, Germany). The titration was terminated with the first appearance of pink colour and the acidity of the sample was calculated as % lactic acid.

Statistical analysis

Collected data was statistically analyzed using analysis of variance by a factorial experiment in a completely randomized design, the experimental factors were whey protein isolate level, storage temperature and time storage. A confidence level of 5% was used to compare means ($p \leq 0.05$) when significance was detected between treatments. The mean values were compared using a Duncan's new Multiple Range Test (DMRT). All the statistical analysis was conducted using SPSS 10.0.1 software (SPSS Inc., Chicago, USA).

Results and discussion

Pasteurization process was applied at 72ºC for 15 s to the imitated milk solution significantly affected the vegetative cells of *B. licheniformis*. A reduction in the TVM count for up to 2.44 log cfu/ml directly after the heating process (Fig. 1) indicating that most of the bacilli vegetative cells suffered heat injuries and the survival cells were dominated by the spore form (Fig. 2), which had a higher heat resistant than the vegetative form. The presence of nisin in the milk solution was further affected the survival of *B. licheniformis* within the first few days of storage. In the absence of whey protein isolate (control treatments), nisin could produced an additional reduction of 0.27 log cfu/ml in the *B. licheniformis* population (Fig. 1). This effect was reduced in the presence of whey protein isolate, 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* was significantly depended on the storage temperature (Fig. 1). In the control treatments, a significant growth of the bacilli at 10ºC storage temperature that reached a microbial population of 4.59 ± 0.03 log cfu/ml was displayed within one week storage followed by a continued growth of the target microorganism until at the end of the storage period. On the other hand, the combination of nisin and a storage temperature of 4ºC would significantly and synergistically inhibit the growth of *B. licheniformis* throughout the studied

storage time. This finding was in an agreement with Montville *et al*. (1995) who reported that the effectiveness of nisin delayed botulinal 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 against *L. monocytogenes* and *S. 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 for *L. monocytogenes*.

The presence of whey protein isolates in the imitated milk solution significantly demonstrated an increased in the antimicrobial effect of nisin against *B. licheniformi*s. Increasing the whey protein isolate 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 synergistic effect of nisin and whey protein isolate needed to be observed at longer storage period. The effectiveness of nisin and pulsed electric fields (PEF) against *Listeria innocua* in whey had also been reported by Gallo *et al*. (2007). Although the report suggested that nisin 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 the 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 the heat injuries, the presence of whey protein isolate 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).

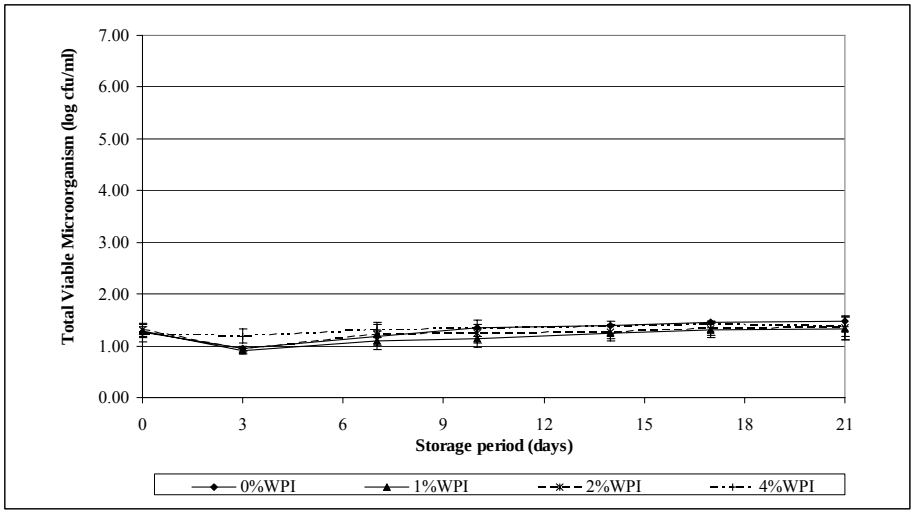
In general, the number of *B. licheniformis* spores had no significant changed throughout 21 days at low storage temperatures. The combination of nisin and refrigerated temperature was sufficient in maintaining the number of the bacilli spores. The presences of whey protein isolate had no any significant 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.

The pH of the imitated milk solution was higher than the normal pH of cow milk, which was within the range of 6.6-6.7 (Walstra *et al*., 1999) (Fig. 3). This higher pH value could be due to the lack of mineral salts addition, such as

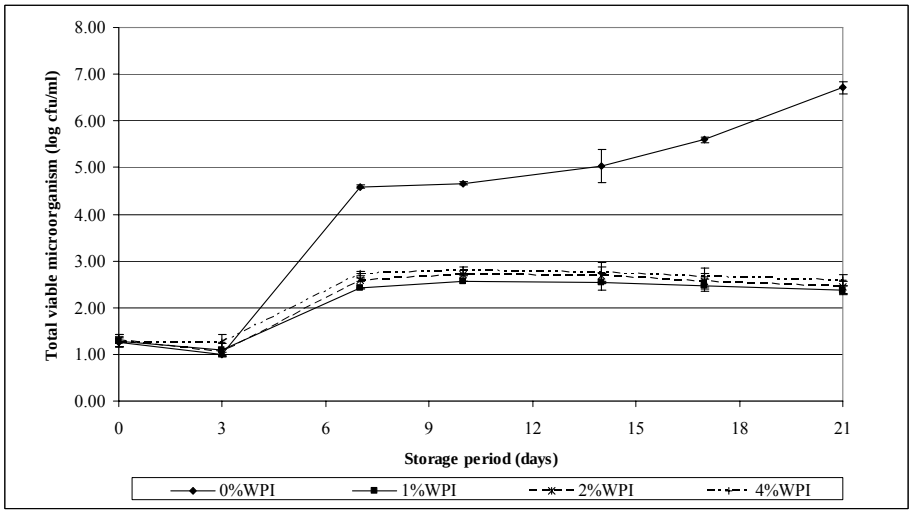
calcium, sodium, phosphate, carbonate and citrate, that affected the ionic balance of the milk solution (Eckner and Zottola, 1992; Walstra *et al*., 1999). During storage at low temperatures, the pH of the milk solutions was significantly reduced together with a significant increase in the total titratable acidity (Fig. 4). 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 (Bridson, 1993). 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 with whey protein isolate. This finding might suggest that the presence of nisin in the milk solution still affected the metabolism activity of *B. licheniformis* eventhough the microorganism itself could increased its population for up to 6.72 ± 0.13 log cfu/ml at the end of the storage period.

Conclusion

Although nisin has been reported to reduce its effectiveness against different target microorganisms in the presence of different food compounds, the result in this study demonstrated that nisin could work synergistically with whey protein isolate in inhibiting the growth of *B. licheniformis* in the imitated milk solution. The effect was significantly more pronounced at 10ºC storage temperature, in which the *B. licheniformis* had a better growth rate than that at 4ºC. The presence of fat, lactose and casein in the milk solution might contribute to the lower effectiveness of nisin against *B. licheniformis* in the control treatment, especially at higher storage temperature. More research would be needed to understand whether the positive antimicrobial effect of nisin and whey protein isolated could be achieved in other food products.

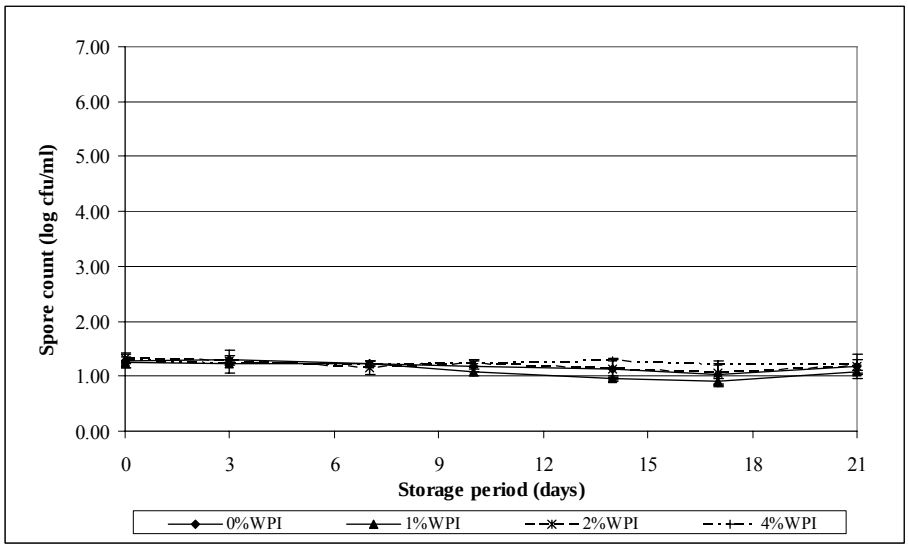


(a)

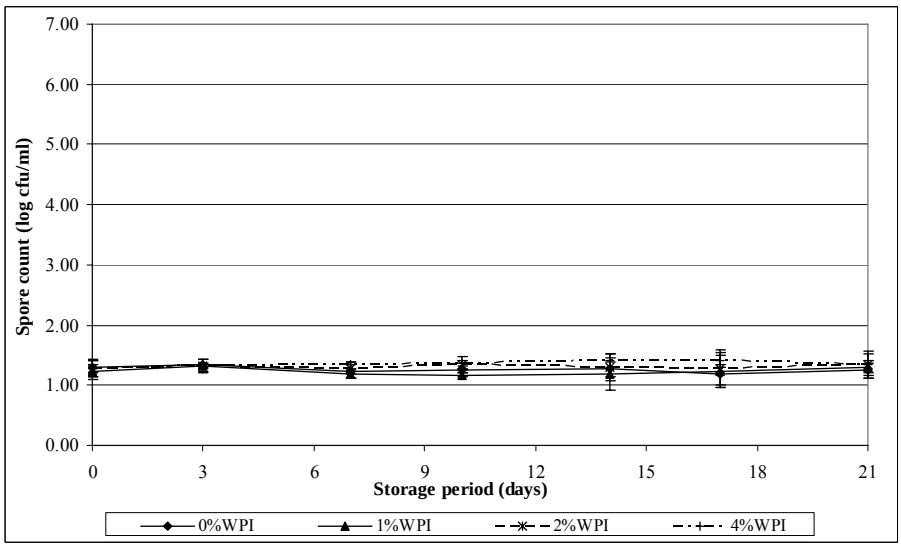


(b)

Fig. 1. Total Viable Microorganism of imitated milk solution affected by different whey protein isolate levels during storage at 4ºC (a) and at 10ºC (b).

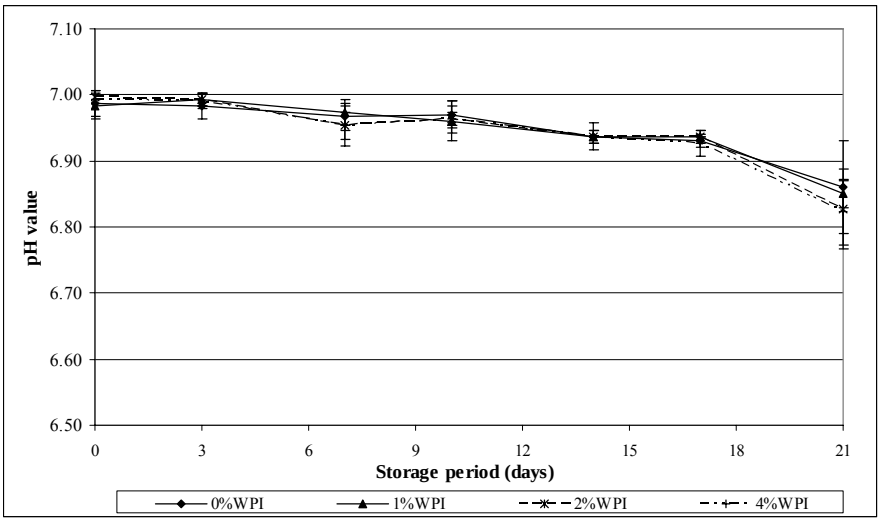


(a)

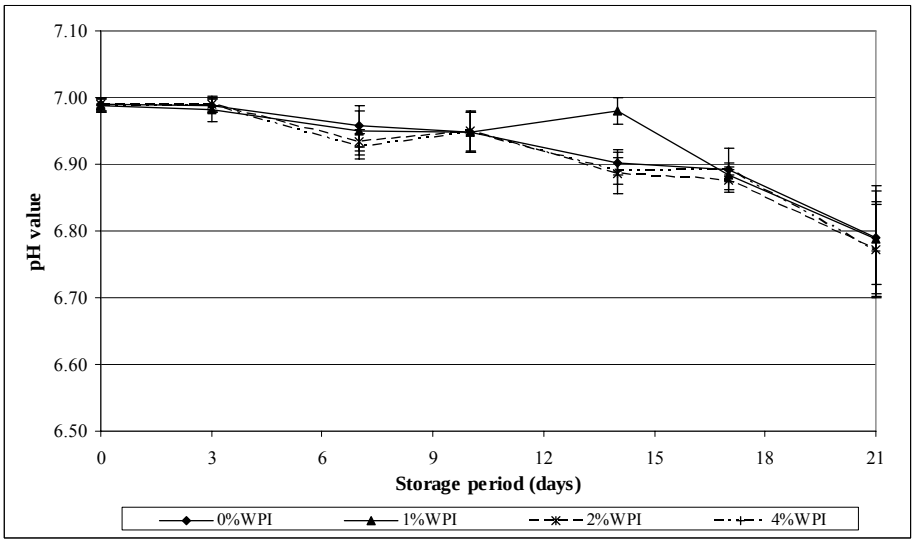


(b)

Fig. 2. Spore counts of imitated milk solution affected by different whey protein isolate levels during storage at 4ºC (a) and at 10ºC (b).

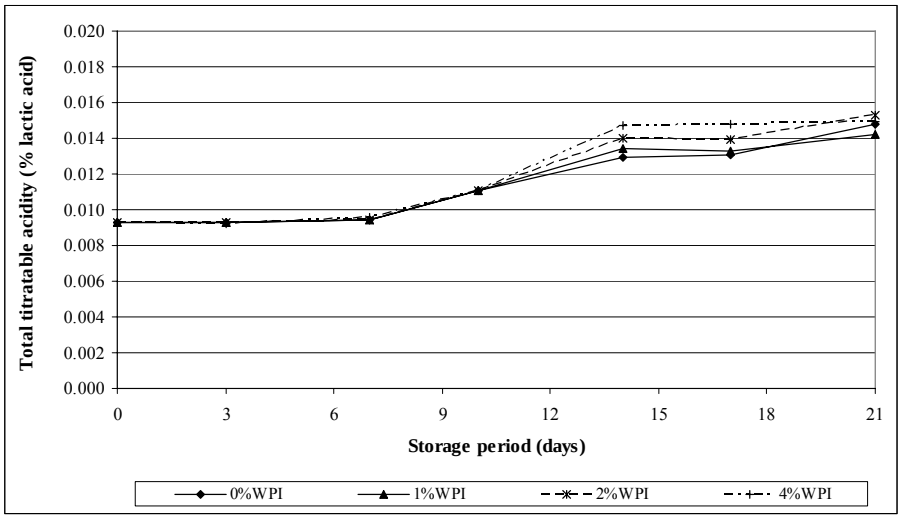


(a)

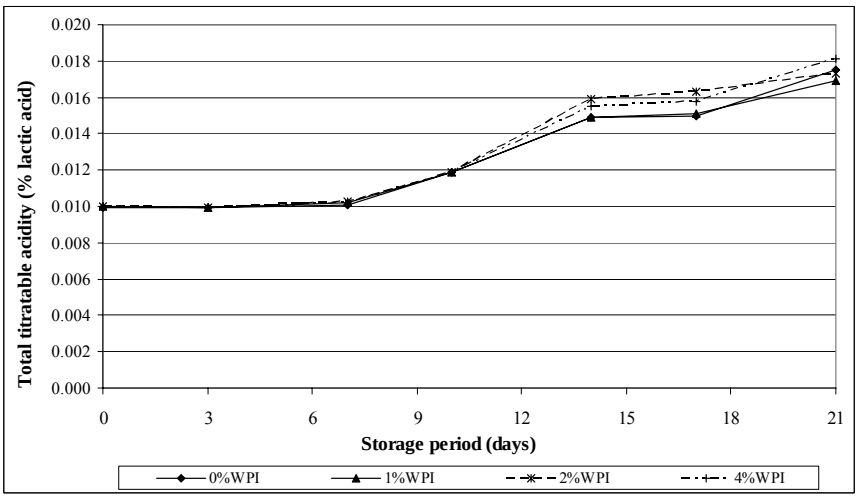


(b)

Fig. 3. pH value of imitated milk solution containing *B. licheniformis* together with the presence of 100 IU/ml nisin and different whey protein isolate levels during storage at 4ºC (a) and at 10ºC (b).



(a)



(b)

Fig. 4. Total titratable acidity (% lactic acid) of imitated milk solution containing *B. licheniformis* together with the presence of 100 IU/ml nisin and different whey protein isolate levels during storage at 4ºC (a) and at 10ºC (b).

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