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Viability of Acid-adapted and Non-adapted *Bacillus cereus* during the Lactic Fermentation of Skim Milk and Product Storage at 5°C

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ABSTRACT

In this study, *Bacillus cereus* was subjected to acid adaptation at pH 6.3 for 40 min. It was found that acid-adapted *B. cereus* exhibited a higher survival percentage than non-adapted cells after exposure to an acidic condition of pH 4.6. The viable population of both acid-adapted and non-adapted *B. cereus* increased during the first 12-18 hr of lactic fermentation of skim milk by *Streptococcus thermophilus* or *Lactobacillus bulgaricus*, and then declined as the fermentation extended. However, the acid-adapted cells exhibited a lower population reduction rate than the non-adapted *B. cereus*. A higher viable population of acid-adapted *B. cereus* than the non-adapted cells was also noted in *Str. thermophilus*-fermented skim milk and the two commercial lactic fermented products kept at 5°C. Additionally, the acid adaptation could reduce the susceptibility of *B. cereus* to acidity, refrigerated temperature and other detrimental principles present in lactic fermented milk products.

Key words: viability, acid adaptation, *Bacillus cereus*, lactic fermented milk, refrigerated storage

INTRODUCTION

Acidification is one of the methods commonly used to control the proliferation of pathogenic microorganisms and spoilage during the preparation and processing of food[1]. Various microorganisms including *Salmonella* spp., *Escherichia coli* O157:H7, *Listeria monocytogennes*, *Vibrio parahaemolyticus* and *Bacillus cereus* have been reported to develop enhanced resistance to higher acidity and other stresses when they have been previously exposed to less severe acidic conditions to induce the induction acid tolerance[2-4]. Moreover, some food-borne pathogens after exposure to a sublethal acid condition have been shown to survive longer in a variety of food systems[5-8]. Therefore, the acid adaptation response of microorganisms has important implications for the food industry where under inappropriate pre-processing storage conditions the pathogenic or spoilage microorganisms may survive the otherwise lethal acidic or other stresses during food preparation and food processing.

B. cereus, a gram positive, facultative anaerobic, and spore-forming bacterium, is widely spread in nature. Many types of food, especially those of plant origin, e.g. rice and pasta are frequently contaminated with this microorganism[9]. It is also reported a common contaminant of raw milk produced on some dairy farms[10]. Various levels of *B. cereus*, ranging from 2 to 52%, were found in dairy products such as ice cream, soft ice cream, milk powder, fermented milk, pasteurized milk and fruit flavored milk[11].

B. cereus may cause flavors to go off and become putrid, rancid, bitter, unclean, fruity and yeasty in fluid milk products[12]. Moreover, Griffiths[13] reported that *B. cereus* caused the defect of sweet curdling in homogenized low-pasteurized milk and estimated that more than 20% of the shelf-life problems encountered with pasteurized milk were due to the proliferation of *B. cereus*. In addition to causing food defects, *B. cereus* may also cause two types of food poisoning, the emetic and diarrhea syndromes and a variety of local and systemic infections[14]. Therefore, the presence of *B. cereus* in foods and food raw materials warrants concern.

Although inhibition of *B. cereus* by various lactic acid bacteria (LAB) (in milk and other media) has been reported[11,15,16], information concerning the effect of acid adaptation on the viability of *B. cereus* during lactic fermentation of milk and product storage remains limited. In the present study, the viability of acid-adapted and non-adapted *B. cereus* was compared during the lactic fermentation of skim milk and product storage at 5°C. Besides, the acid adaptation response of *B. cereus*

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that leads to different viability of acid-adapted and non-adapted cells of test organism during refrigerated storage was also investigated.

MATERIALS AND METHODS

I. *Bacterium and Preparation of Inoculum*

B. cereus BCRC 11837 and lactic acid bacteria (LAB) including *Streptococcus thermophilus* BCRC 14085 and *Lactobacillus bulgaricus* BCRC 10696 were obtained from Bioresources Collection and Research Center, Hsinchu, Taiwan.

B. cereus was activated by subculturing three times in tryptic soy broth (TSB, pH 7.3, Difco, Sparks, MD, USA) at 30°C for 12 hr. The activated culture, containing *B. cereus* at a population of *ca.* 10^8 CFU/mL, was then used to prepare inocula of acid-adapted and non-adapted *B. cereus*.

To prepare the inoculum of LAB, culture of either *Str. thermophilus* or *L. bulgaricus* were subcultured twice in *Lactobacillus* MRS broth (Difico) at 37°C for 18 hr. The activated culture was then inoculated into skim milk broth (SMB) consisting of 13% instant skim milk powder (Anchor, Wellington, New Zealand) and 1% sucrose. After incubation at 37°C for 18 hr, it was diluted with peptone water (Difco) and was used as the inoculum of LAB.

II. *Acid Adaptation of B. cereus*

Acid adaptation of *B. cereus* cells was performed according to the procedures described by Browne and Dowd's(3). In brief, the activated culture of *B. cereus* was first centrifuged at 6,000 rpm for 20 min. The cell pellets were washed with phosphate buffer solution (PBS, pH 7.0) twice, resuspended in PBS (non-adapted) or PBS whose pH was adjusted to 6.3 with 1 N HCl and incubated at room temperature (22-25°C) for 40 min. After dilution with saline solution, these cultures were used as the inoculum of non-adapted or acid-adapted *B. cereus*.

III. *Culture Conditions*

To perform fermentation, 50 mL of SMB supplemented with 1% sucrose, was placed in a 125-mL screw-cap Erlenmeyer flask and autoclaved at 121°C for 15 min. It was then inoculated with either a single strain of acid-adapted, non-adapted *B. cereus*, *Str. thermophilus* BCRC 14085 or *L. bulgaricus* BCRC 10696, or was inoculated simultaneously with both single strains of acid-adapted or non-adapted *B. cereus* and *Str. thermophilus* or *L. bulgaricus*. The initial population of *B. cereus* and lactic acid bacteria was controlled at *ca.* 10^{6-7} CFU/mL. These cultures were incubated quiescently at 37°C for a period of 48 hr. During the fermentation period, samples were taken at predetermined intervals to determine the titratable acidity, pH, and viable populations of *B. cereus* and lactic acid bacteria.

IV. *Storage Study in Laboratory-prepared and Commercial Lactic Fermented Products at 5°C*

In this study, the *Str. thermophilus*-fermented milk, prepared as described previously, and two commercial fermented milk products, yoghurt A (pH 4.20) and yoghurt B (pH 4.23) from a local supermarket, were examined. Each lactic fermented product (50 mL) was inoculated with the prepared acid-adapted or non-adapted *B. cereus* to achieve an initial population of *ca.* 6.0 log CFU/mL. They were then stored at 5°C for 10 days. During the storage period, the viable population of *B. cereus* was examined periodically as specified in Results and Discussion.

V. *Storage Study in the Treated-lactic Fermented Products and SMB at 5°C*

This study was aimed to examine whether acid adaptation affected the susceptibility of *B. cereus* to refrigerated temperatures or other detrimental factors other than pH. The *Str. thermophilus*-fermented skim milk and commercial yoghurt A were first centrifuged at 10,000 rpm for 20 min at 4°C. Supernatant fractions were adjusted to pH 6.4, which is the pH of the prepared SMB, with 1.0 N NaOH solution and filtered through a membrane filter. Acid-adapted or non-adapted cells of *B. cereus* were inoculated into the treated-fermented milk products and SMB (pH 6.4) at an initial concentration of ca 6.0 log CFU/mL and kept at 5°C for 10 days. The viable population of *B. cereus* was determined at the end of the storage.

VI. *Microbiological and Chemical Analysis*

For the enumeration of test organisms, samples were first serially diluted with saline solution. To determine the viable population of *B. cereus*, 1.0 mL of the serially diluted samples were pour-plated on tryptic soy agar (TSA, Difco). Colonies on the plates were counted after 24 hr of incubation at 30°C. In the lactic fermentation and storage study, Mannitol egg yolk polymyxin agar (Difco) instead of TSA was used as plating medium(17). Colonies were counted after incubation at 37°C for 24 hr. To enumerate lactic acid bacteria, 1 mL of the serially diluted samples were pour-plated on MRS agar (Difco). Colonies on the plate were counted after 48 hr of incubation at 30°C.

The pH of the samples was measured using a pH meter. Titratable acidity was determined according to the method of AOAC(18) and expressed in terms of lactic acid content (%).

VII. *Statistical Analysis*

The mean values and the standard deviation were calculated from the data obtained from three separate experiments. These data were then compared using the Duncan's multiple range test (SAS, 2001)[19].

RESULTS AND DISCUSSION

I. *Effect of Acid Adaptation on the Survival of B. cereus in Phosphate Buffer (pH 4.6)*

The survival behaviors of the acid-adapted and the non-adapted *B. cereus* cells in PBS (pH 4.6) are shown in Figure 1. It was found that the viable population of the non-adapted *B. cereus* declined rapidly during the first 10 min of exposure. At the end of the 30-min exposure, the non-adapted *B. cereus* reached a viable population of 2.37 log CFU/mL. On the other hand, the reduction of the viable population for the acid-adapted *B. cereus*, especially during the initial exposure period of 15 min, was relatively milder than that of the non-adapted cells. After 15 min of exposure, the acid adapted *B. cereus* exhibited a viable population of 4.89 log CFU/mL which is significantly higher ($P < 0.05$) than that of the non-adapted one (2.61 log CFU/mL). Probably due to strain difference, the extent of acid tolerance enhancing effect observed on *B. cereus* in this study is less than that reported for *B. cereus* NCIMB 11796[3]. They observed that the survival rate of acid-adapted cells was higher than that of non-adapted cells by 3.0-6.0 log CFU/mL. Data shown in Figure 1 demonstrated that the acid treatment did enhance the acid tolerance of *B. cereus*.

II. *Titritable Acidity and pH of Skim Milk during the Fermentation with Acid-adapted or Non-adapted B. cereus and with or without Lactic Acid Bacteria*

As shown in Figure 2A, changes of titritable acidity and pH of skim milk caused by the acid-adapted or the non-adapted *B. cereus* were both minor during the fermentation period. The pH of skim milk with *B. cereus*, regardless of acid adaptation, decreased from ca 6.41-6.42 to 5.14-5.15, while titritable acidity increased from 0.19 to 0.56-0.58% at the end of fermentation. On the other hand, a marked change in pH and titritable acidity was noted in skim milk containing lactic acid bacteria and acid-adapted or non-adapted *B. cereus* (Figure 2 B and C). For example, titritable acidity of skim milk inoculated with either acid-adapted or non-adapted *B. cereus* and *S. thermophilus* increased from 0.19 to 1.14-1.15%, while pH decreased from 6.26-6.27 to 4.12-4.14 after 48 hr of fermentation (Figure 2B). A similar phenomenon was also observed in skim milk fermented simultaneously with either acid-adapted or non-adapted *B. cereus* and *L. bulgaricus* (Figure 2C). The marked increase in

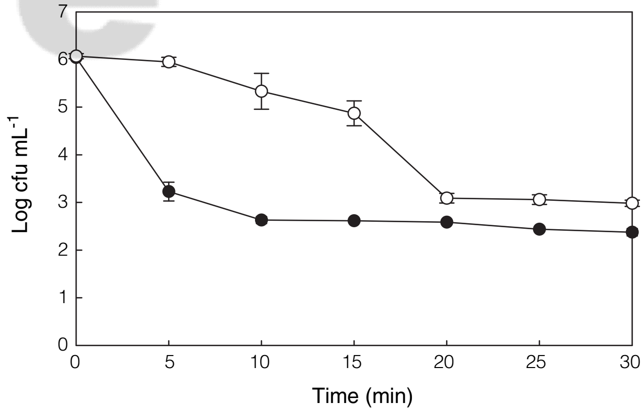

Figure 1. Effect of acid-adaptation on the survival of *B. cereus* after subsequent exposure to phosphate buffer (pH 4.6). ○, acid-adapted cells; ●, non-adapted cells. The initial populations of the control and acid-adapted *B. cereus* were *ca.* 10^6 CFU/mL.

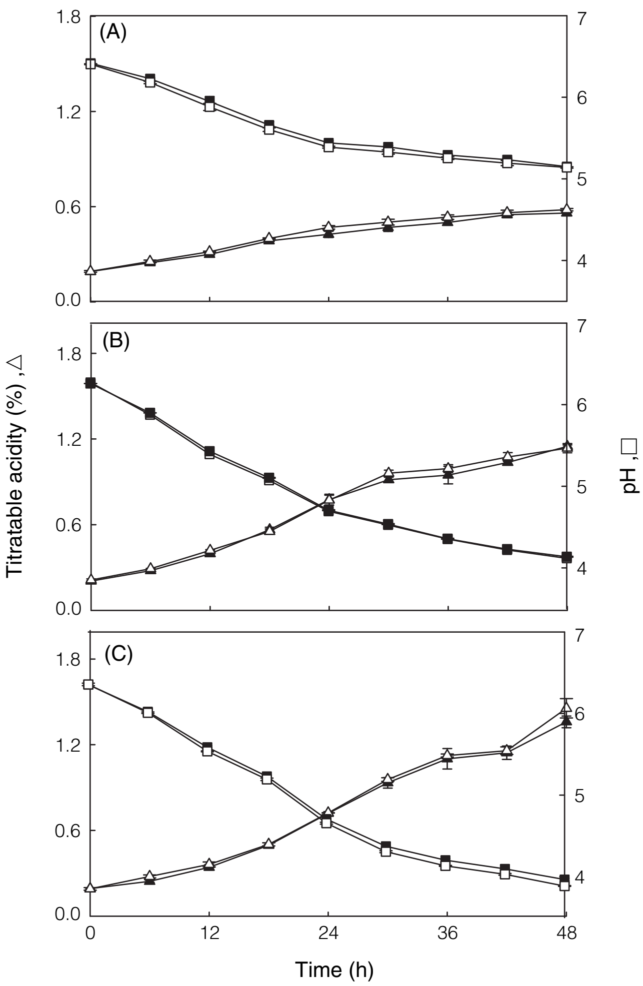

Figure 2. Change of titratable acidity and pH in skim milk during the cultivation of acid-adapted or non-adapted *B. cereus* alone and with or without lactic acid bacteria. Triangle symbol, titratable acidity; square symbol, pH. (A) Skim milk inoculated with *B. cereus* alone; (B) Skim milk inoculated with *B. cereus* and *Str. thermophilus*; (C) Skim milk inoculated with *B. cereus* and *L. bulgaricus*. △,□, acid-adapted cells; ▲,■,non-adapted cells.

titritable acidity and reduced pH observed in these LAB-containing skim milk was apparently attributed to the growth of LAB. LAB with a population of *ca.* 9.0 log CFU/mL was observed in the skim milk after 48 hr of fermentation. It was also noted that skim milk fermented simultaneously with LAB and either acid-adapted or non-adapted *B. cereus* showed no marked difference in the change of pH and titritable acidity during fermentation (Figure 2 B and C). This demonstrated that presence of *B. cereus*, regardless of acid adaptation, did not affect the change of pH and acidity of skim milk during lactic fermentation.

III. *Behavior of Acid-adapted and Non-adapted B. cereus in Skim Milk with or without Lactic Acid Bacteria*

The growth and survival of acid-adapted and non-adapted *B. cereus* in skim milk inoculated with or without LAB are shown in Figure 3. Growth of the pure culture of acid-adapted and non-adapted *B. cereus* in skim milk showed no difference (Figure 3A). Both acid-adapted and non-adapted *B. cereus* grew and reached their stationary phase with a population of ca 8.41-8.67 log CFU/mL after 18 hr of cultivation. The viable populations remained rather constant throughout the rest of the 48 hr of fermentation.

The growth pattern of acid-adapted and non-adapted *B. cereus* inoculated with lactic acid bacteria during the first 12-18 hr of cultivation (Figure 3 B and C) was similar to those observed in skim milk inoculated with *B. cereus* alone (Figure 3A). While, a reduction in the viable population of *B. cereus* was noted as the fermentation continued. This phenomenon is similar to that reported by R ssland *et al.*[16]. However, we noted that the reduction rate was lower with the acid-adapted than with the non-adapted *B. cereus*. At the end of 48-hr of fermentation, the acid-adapted *B. cereus* showed a significantly higher ($P < 0.05$) viable population of 4.9 log CFU/mL than that of 2.7 log CFU/mL found with the non-adapted cells in the skim milk containing *S. thermophilus* (Figure 3B). Meanwhile, the acid-adapted *B. cereus* showed a viable population of 3.20 log CFU/mL, while the non-adapted cell became undetectable in the *L. bulgaricus*-containing skim milk (Figure 3C). This experiment demonstrated that acid adaptation did enhance the survival of *B. cereus* during the lactic fermentation of skim milk.

As shown in Figure 2, skim milk containing lactic acid bacteria exhibited a higher titritable acidity and a lower pH than did the skim milk alone after 12-18 hr of fermentation. The pH continued to drop while the titritable acidity continued to increase in the skim milk containing lactic acid bacteria as fermentation further extended and reached to levels of 3.89-4.14 and 1.14-1.45%, respectively, at the end of fermentation (Figure 2 B and C). The high acidity noted in the LAB-containing skim milk may cause contribute to the marked reduction in the viable population of *B. cereus*, regardless of acid adaptation[16]. However, we can not rule out the possibility that other detrimental factors such as hydrogen peroxide and ethanol produced by lactic bacteria[20,21] may also lead to the suppression of the viable population of *B. cereus*.

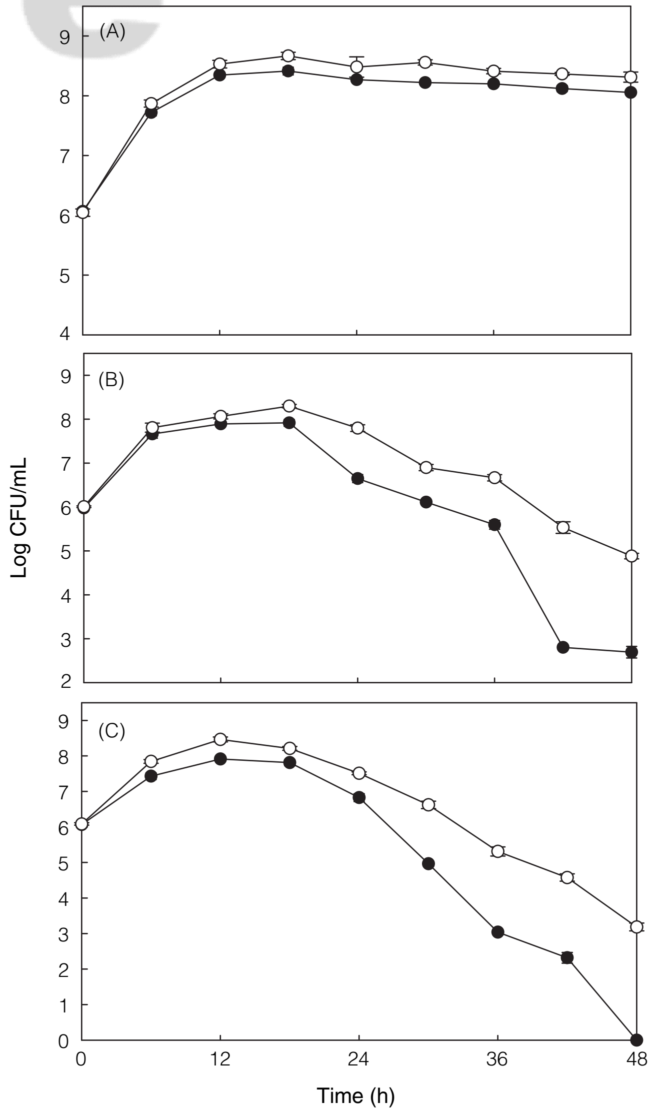

Figure 3. Growth behavior of acid-adapted and non-adapted *B. cereus* in skim milk inoculated with or without lactic acid bacteria. (A) Skim milk inoculated with *B. cereus* alone; (B) Skim milk inoculated with *B. cereus* and *Str. thermophilus*; (C) Skim milk inoculated with *B. cereus* and *L. bulgaricus*. ○,acid-adapted cells; ●, non-adapted cells.

IV. *Growth Behavior of Lactic Acid Bacteria in Skim Milk with or without B. cereus*

The changes in the viable population of LAB in skim milk inoculated simultaneously with or without *B. cereus* are shown in Figure 4. Regardless of an inoculation of *B. cereus*, the viable population of *Str. thermophilus* increased rapidly in skim milk during the first 8 hr of fermentation, increased slowly as the cultivation was further extended, and reached a population of *ca.* 9.0 log CFU/mL at the end (Figure 4A). On the other hand, the presence of acid-adapted or non-adapted *B. cereus* seemed to enhance the growth of *L. bulgaricus*. After 24-hr fermentation, the viable population of *L. bulgaricus* was higher in the *B. cereus*-containing skim milk than that in the skim milk without *B. cereus* (Figure 4B). A similar phenomenon was observed by Gonz lez *et al*.[22] who reported that growth rates of *L. casei* ssp *casei* or *L. acidophilus* determined at the 6th hour of cultivation was higher in mixed cultures with enteropathogenic *E. coli* than that in the pure culture. Furthermore, as shown in Figure 4B, the viable population of *L. bulgaricus* noted in skim milk inoculated with acid-adapted *B. cereus* was found close to that in skim milk containing non-adapted *B. cereus*.

V. *Behavior of Acid-adapted and Non-adapted B. cereus in Lactic Fermented Product of Milk Kept at 5°C*

Acid adaptation enhancing the survival of food-borne pathogens such as *Sal. thphimurium*, *E. coli* O157: H7 and *Listeria monocytogenes* in various food systems has been observed[5,7,8,23]. It has also been reported that acid adaptation increased the survival of *E. coli* O157:H7 in acidic fruit juice during storage. And yet the survival of the acid-adapted counterpart *E. coli* O157:H7 in lactic fermented products remains to be examined.

The survival behaviors of the acid-adapted and non-adapted *B. cereus* in the *Str. thermophilus*-fermented skim milk prepared in our laboratory and two commercial lactic fermented milk products during 10-days of storage at 5°C are shown in Figure 5. Regardless of acid adaptation, the viable population of *B. cereus* in all the lactic fermented products decreased rapidly during the first 1-2 day of storage at 5°C. No viable cells of acid-adapted or non-adapted *B. cereus* were detected in commercial yoghurt A (Figure 5B) while the *Str. thermophilus*-fermented skim milk and commercial yoghurt B still contained viable *B. cereus* with a population of 1.97-2.15 and 2.21-2.38 log CFU/mL, respectively, at

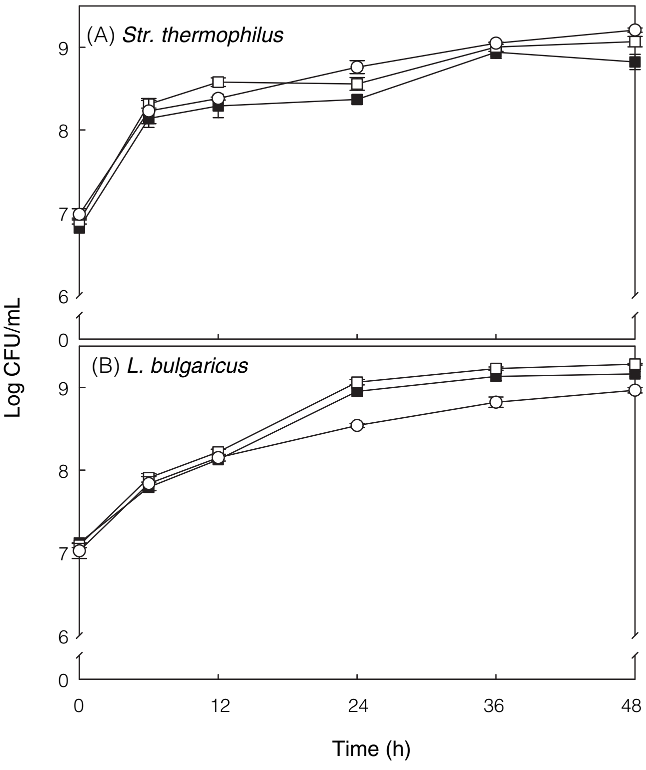

Figure 4. Growth of (A) *Str. thermophilus* or (B) *L. bulgaricus* in skim milk with or without *B. cereus*. Skim milk inoculated with non-adapted *B. cereus* and lactic acid bacteria, ■; skim milk inoculated with acid-adapted *B. cereus* and lactic acid bacteria, □; skim milk inoculated with lactic acid bacteria only, ○.

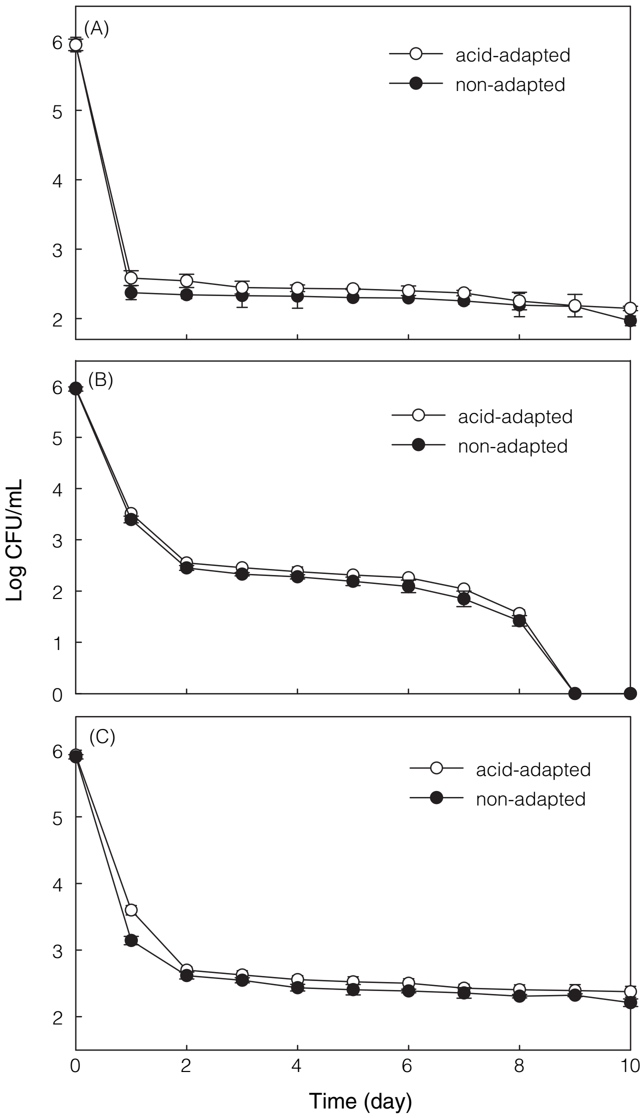

Figure 5. Survival of acid-adapted and nonadapted *B. cereus* in (A) *Str. thermophilus*- fermented skim milk and (B) Commercial yoghurt A, (C) Commercial yoghurt B during storage at 5°C.

the end of 10-day storage (Figure 5 A and C). Difference in the viable population of test organism observed in the lactic fermented milk products during storage may be attributed various of starter organism used for these fermented products. Besides, various added ingredients by manufacturers may also cause these discrepancies. However, the exact reason remained to be explored. As shown in Figure 5, the viable population of the acid-adapted was, generally, higher than that of the non-adapted cells as determined at various storage periods. The most marked difference in the viable population of acid-adapted and non-adapted cells was noted in commercial yoghurt B after 1 day of storage. Estimating the population reduction by subtracting the final population (log CFU/mL) from the initial population of test organism also revealed that the acid-adapted cells, in general, exhibited a lower population reduction than did the non-adapted ones. For example, the acid-adapted *B. cereus* at the end of storage exhibited a population reduction of log 3.55 CFU/mL which is significantly lower ($P < 0.05$) than that (log 3.69 CFU/mL) detected in the non-adapted cells in commercial yoghurt B (Figure 5C).

VI. *Survival of Acid-adapted and Non-adapted B. cerues in Skim Milk and the Treated- lactic Fermented Product of Milk Kept at 5°C*

Refrigeration and the metabolites of lactics (e.g. ethanol, bacteriocin, hydrogen peroxide and diacetyl), are detrimental to microorganisms[20,21]. These detrimental factors, in addition to acidity, may also contribute to the reduction of the viable *B. cereus* cells in the lactic fermented milk products kept at 5°C as observed in the present study (Figure 5). An attempt was made to examine whether the acid adaptation also affects the susceptibility of test organism to these detrimental factors by comparing the survival of the acid-adapted and non-adapted *B. cereus* in cell-free fermented milk (pH adjusted to 6.4) and skim milk (pH 6.4) kept at 5°C. In this experimental design, acidity was no longer a detrimental factor to the test organism. As shown in Table 1, acid-adapted *B. cereus* exhibited a significantly lower ($P < 0.05$) population reduction than did the non-adapted cell in skim milk kept at 5°C, which supposedly contained no antimicrobial principles. This implied that acid adaptation may reduce the susceptibility of test organism to refrigeration. A reduction in the viable population of acid-adapted and non-adapted *B. cereus* was also noted in the treated-lactic fermented products. The higher population reduction of the acid-adapted and non-adapted *B. cereus* noted in the treated-lactic fermented product compared to the *B. cereus* in skim milk suggested the possible role of other detrimental factors such as antimicrobial metabolites of lactic acid bacteria. Additionally, the higher population reduction of the non-adapted cells compared to the acid-adapted *B. cereus* noted in the treated-lactic fermented products further suggests that acid adaptation may also reduce the susceptibility of *B. cereus* to the antimicrobial metabolites of lactics. These observations are consistent with reports of Leyer and Johnson[2] and Tosun and Gonul[24]. They demonstrated that acid adaptation induced cross-protection against environmental stresses in *Sal. typhimurium*.

Table 1. Survival of acid-adapted and non-adapted *B. cereus* in the treated lactic fermented milk and skim milk after storage at 5°C for 10 day

	Initial population (Log CFU/mL)	Final population (Log CFU/mL)	Population reductiona (Log CFU/mL)
Skim milk			
nonadapted	6.04 ± 0.07	3.30 ± 0.06	2.75 ± 0.09 A[c]
acid-adapted	6.06 ± 0.07	4.66 ± 0.10	1.39 ± 0.04 B
Treated commercial yoghurt Ab			
nonadapted	6.02 ± 0.04	2.47 ± 0.07	3.55 ± 0.08 A
acid-adapted	6.06 ± 0.02	2.99 ± 0.05	3.07 ± 0.07 B
Treated *Str. thermophilus*– skim fermented milk[b]			
nonadapted	6.04 ± 0.08	2.55 ± 0.07	3.49 ± 0.05 A
acid-adapted	6.03 ± 0.06	3.39 ± 0.02	2.64 ± 0.04 B

[a]Population reduction was obtained by subtracting final population (Log CFU/mL) from initial population (Log CFU/mL).
[b]The lactic fermented milk were centrifuged for 20 min at 10,000 rpm Supernatant fractions were adjusted to pH 6.4, then filtered through 0.22 m membrane.
[c]Value in the same column for same milk product with different upper case letters are significantly different ($p < 0.05$).

CONCLUSIONS

Results obtained from the present study showed that acid adaptation increased the resistance of *B. cereus* to acid, refrigeration, and possible antimicrobial metabolites of LAB present in lactic fermented milk products. It follows that acid adaptation likely contributes to the enhanced survival rate of acid-adapted *B. cereus* as noted in the present study. Nevertheless, *B. cereus*, regardless of acid adaptation, is capable of growing during lactic fermentation and can persist in the lactic fermented milk for more than 10 days of refrigerated storage. This has important implications for food safety. Adequate standards of hygiene must be enforced to avoid the contaminations of *B. cereus* during the preparation and storage of fermented milk products so that the incidence of food deterioration and food-borne illness caused by this pathogen can be reduced.

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