

Impact of temperature on *Bacillus cereus* spore germination in ultra-high temperature chocolate milk

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Abstract

Bacillus cereus spores are capable of surviving the harsh environment and more often, they cause great concern to the dairy industry. The current research was conducted to study the effect of temperature on germination and growth of *B. cereus* spores in UHT chocolate milk; the study was carried out at 8°C, 25°C and 35°C over a span of seven days. The results showed that no growth was observed at 8°C. At 25°C, a rapid increase in growth was observed as early as Day 1, from an initial count of ten spores to 4.01 log₁₀ CFU/mL. Meanwhile, at 35°C, the growth on Day 1 was more rapid in which the count promptly increased to 8.07 log₁₀ CFU/mL. Analysis of graph trend showed that the number of vegetative cells decreased while the number of spores increased with incubation time due to nutrients exhaustion. This study fills up the data gap towards understanding the possible issues that might arise in the actual scenario and at the same time, suggests a suitable approach to minimize infection risk caused by *B. cereus* spores.

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1. Introduction

Bacillus and *Clostridium* are the known bacterial genera that are capable of producing spores under hostile environment. They thrive in the unfavourable conditions by producing endospores that protect the organism's main DNA and for replication purpose when the endospores germinate. An endospore is dormant, durable, and it does not carry out the metabolic reaction. Thanks to their durable nature, endospores are resistant to extreme heat, lack of water, exposure to various kind of toxic chemicals and radiation. Not to mention, they could sustain well without nutrients for a long period of time. It was previously described by Tortora *et al.* (2006) in their article that spores could persist in the environment for millions of years.

Bacillus cereus is a foodborne disease agent that is considered a high surveillance bacterium in the food manufacturing process, especially dairy products. The reintroduction of *B. cereus* in food is fairly low due to its non-competitive nature (Granum and Lund, 1997). However, the transfer of its endospores into food is the main transmission route of *B. cereus* as the endospores can be present anywhere due to its saprophytic life cycle; germinating, growing and sporulating within the

environment (Vilain *et al.*, 2006). From a clinical and food industry point of view, endospores are important due to their resistance towards many processes that normally kill vegetative cells. The processes include heating, freezing, desiccation, use of chemicals and radiation. When describing their extreme resistance, the endospores could survive sterilization of surgical equipment and in the same manner, surviving the heat treatment on food products, for example, pasteurisation and ultra-high heat treatment.

Endospore forms when there are triggers such as lack of key nutrients such as the carbon and nitrogen source. The process of endospore formation is known as sporulation or sporogenesis. *Bacillus cereus* sporogenesis begins with replication of chromosomal DNA and isolation of a small portion of cytoplasm by in growth of plasma membrane which eventually forms the forespore. Next, thick peptidoglycan layers will cover the forespore, followed by thick spore coat which responsible for endospore's resistance. At the end of sporogenesis, the original cell degrades, and endospore is released into the environment. It will eventually resume its vegetative state by a process known as germination when the environment becomes favourable again (Setlow, 2014).

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Essentially, there are two main categories in which microbial growth requirements can be divided into: physical and chemical. The physical categories include temperature, pH and osmotic pressure; chemical aspects include sources of carbon, nitrogen, sulphur, phosphorus, trace elements, oxygen and organic growth factors (Tortora *et al.*, 2006). Temperature is the most implicated on microbial growth. It was noted by Camper *et al.* (1999), most regrowth events of the microbial population occur with the rise of temperature, which is an inevitable issue in Asian countries due to the climate.

Bacillus cereus is classified as mesophiles whereby their optimum temperature range falls within 30°C to 37°C. Some strains were reported to grow well after cooking and cooling (< 50°C) (Granum and Lund, 1997; IDF, 2016). According to Lechner *et al.* (1998), *B. weihenstephanensis* was the psychrotolerant species within the *B. cereus* group due to its ability to grow below 7°C, identified via its specific signature sequences in 16S rRNA and cold-shock protein genes. In addition, depending on genetics, there are some *B. cereus* strains that have the ability to grow below 4°C (IDF, 2016).

Despite that, there are contributing factors aforementioned which may affect the growth rate of *B. cereus* population. This present study will investigate the impact of temperature on the germination of *B. cereus* spores in UHT chocolate milk. The study focused on the *B. cereus* endospores as UHT is a process designed to destroy almost all vegetative cells of microorganism present in the sample product but unable to inactivate endospores.

2. Materials and methods

2.1 Bacterial strain preparation

Bacillus cereus ATCC 33019 strain used in this study was obtained from the American Type Culture Collection (Rockville, MD, USA). *Bacillus cereus* was revived and stored in glycerol stocks. From the glycerol stock, a loopful of *B. cereus* ATCC 33019 was inoculated into Tryptic Soy Broth (TSB) and incubated at 37°C for 24 hrs.

2.2 Preparation of spore suspension

Preparation of spore suspension was done according to the method described by Giffel *et al.* (1995) with modifications according to Rukayadi *et al.* (2009). Sporulation was achieved by inoculating a 24-hour culture of *B. cereus* in TSB onto nutrient agar. The culture was incubated at 30°C for a week. After one-week incubation, spores were washed from agar surface with sterile distilled water and collected in a 15 mL centrifuge tube. The suspension was centrifuged at 2000

x g for 20 mins and the supernatant was discarded. Following that, the pellet was washed three times and resuspended with sterile distilled water. In order to destroy vegetative cells, the spore suspension was subjected to heat shock of 80°C for 15 mins. The number of spores was determined using the plate count method using plate count agar. The prepared spore suspension was used immediately in this study.

2.3 Spore germination study

A total of two packs of sterile UHT chocolate milk were used in this study. A pack was spiked with approximately 10 spores/ml using a sterile syringe, following that, the punctured hole was sealed using hot glue. Another pack was kept sterile and served as control. The UHT chocolate milk packs were incubated for 7 days at 8°C, 25°C and 35°C; 8°C – to simulate milk that were kept in refrigerator, 25°C – to simulate the packs being kept at room temperature on retail stores' display shelves and 35°C – to simulate temperature abuse.

To count vegetative cells and spores' growth after 24 hrs, 1 mL of UHT chocolate milk from each pack was serially diluted with sterile distilled water and plated onto plate count agar. For spores' growth, one ml of UHT chocolate milk was subjected to a heat shock of 80°C for 15 mins. Following that, serial dilution for heat-shocked chocolate milk was performed as previously mentioned. Plate count agars were then incubated at 37°C for 15 hrs. *Bacillus cereus* colonies were counted using colony counter (Galaxy 230 Colony Counter, Taiwan). Spores germination were analysed for every 24 hrs for seven days.

3. Results and discussion

At the beginning of the study, UHT chocolate milk was first subjected to milk sterility check. One ml aliquot from UHT chocolate milk was added to 9 mL TSB and the solution was mixed thoroughly using vortex. The mixture was incubated at 37°C for 48 hrs. After 48 hrs incubation, the mixture was observed for sterility validation; the presence of foul odour, curdling and changes in chocolate milk colour. For all samples purchased, no spoilage was observed.

The study was carried out at three different temperatures; (1) at refrigeration temperature to simulate the milk being placed in household refrigerators; (2) at room temperature to simulate the room temperature condition in which the milk was being displayed at air-conditioned retail stores and; (3) at a temperature when the milk is placed under non-air-conditioned storage places where the milk could be subjected to temperature

abuse. Figures 1 and 2 show the germination and growth of *B. cereus* spores within the span of seven days. *Bacillus cereus* growth can be divided into two parts: (1) growth of vegetative cells and (2) number of spores formed during the whole incubation time.

At 8°C, there was no growth observed for seven days of study for both spores and vegetative cells. Figure 1 shows a constant number of spores spiked From Day 1 until Day 7. The number of spores remained constant. Sensory evaluations were performed on chocolate milk; milk texture was smooth as no curdling observed. Milk pack was not bloated and there was no foul smell detected.

In the earlier stage of analysis for temperature 25°C (Figure 2), vegetative cells showed a rapid increase in growth on Day 1, from 10 spores to 4.01 log₁₀ CFU/mL and subsequently doubled on Day 2 (8.42 log₁₀ CFU/mL). Throughout Day 3 to Day 7, the growth began to decrease slowly in number (from 8.47 log₁₀ CFU/mL to 7.30 log₁₀ CFU/mL) as it was reaching stationary phase. Simultaneously, the number of spores began to increase starting on Day 3 (4.43 log₁₀ CFU/mL) and gradually increased in number throughout Day 4, Day 5 and Day 6 (4.77 log₁₀ CFU/mL, 5.03 log₁₀ CFU/mL and 5.44 log₁₀ CFU/mL respectively). A number of spores began to decrease on Day 7 (5.34 log₁₀ CFU/mL) as it reached the stationary phase. Curding was observed in milk texture;

milk pack showed bloating starting Day 1 and there was foul smell detected.

Figure 3 shows the results for analysis on temperature 35°C. Vegetative cells showed rapid growth as early as Day 1, starting from 10 spores and promptly increased to 8.07 log₁₀ CFU/mL. The count increased slightly to 8.24 log₁₀ CFU/mL on Day 2. However, the number of vegetative cells started to decline gradually on Day 3 (7.49 log₁₀ CFU/mL) and Day 4 (7.45 log₁₀ CFU/mL). They slightly increased on Day 5 (7.46 log₁₀ CFU/mL) as well as Day 6 (7.65 log₁₀ CFU/mL) and finally, they decrease again on Day 7 (7.49 log₁₀ CFU/mL) as it reached stationary phase. The number of spores began to increase on Day 2 (5.83 log₁₀ CFU/mL) and they continue to moderately increase on Day 3 throughout Day 7 (from 6.27 log₁₀ CFU/mL to 6.66 log₁₀ CFU/mL). There was a very slight decrease of spore number on Day 5 (6.20 log₁₀ CFU/mL) before they increase again on Day 6 to Day 7. Curding was also observed in milk texture; milk pack also showed bloating starting Day 1 and there was also foul smell detected.

At 8°C, no spore germination and growth were detected during analysis, in which this temperature is distinctly not the optimum growth temperature for *B. cereus*. Hence, the milk remained unaltered. At the end of the analysis (Day 7), the milk was then transferred to 35°C incubator; spores resumed germination and growth

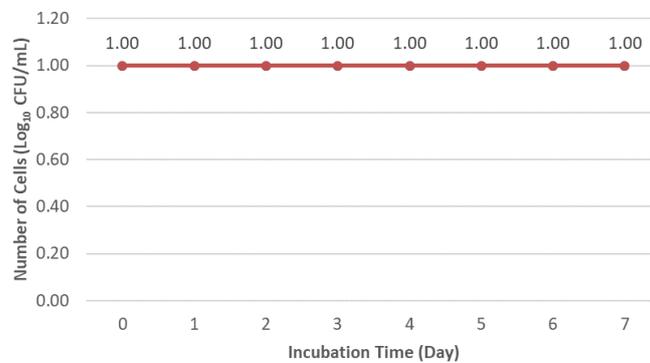


Figure 1. Growth curve at 8°C showing the growth of vegetative cells and spores both indicated with the red line in a seven-day study.

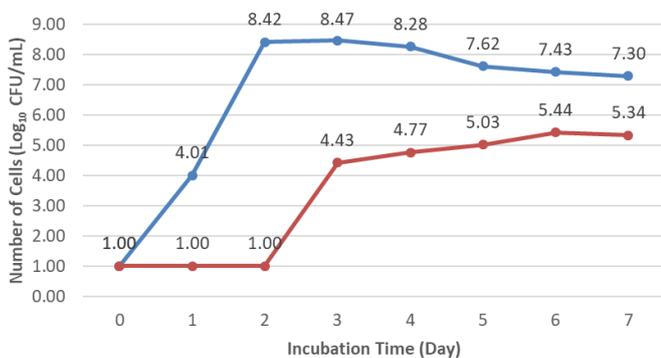


Figure 2. Growth curve at 25°C showing (1) germination and growth of vegetative cells indicated with the blue line and (2) formation of spores indicated with the red line in a seven-day study.

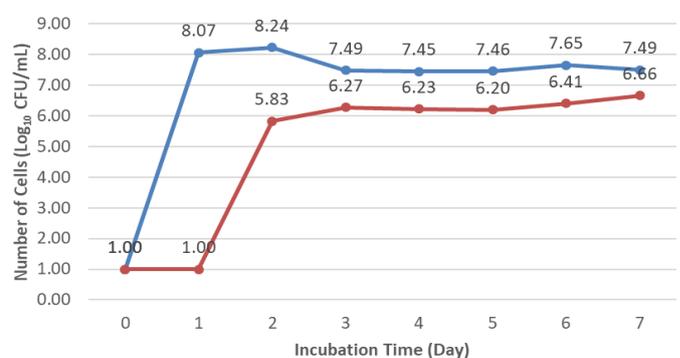


Figure 3. Growth curve at 35°C showing (1) germination and growth of vegetative cells indicated with the blue line and (2) formation of spores indicated with the red line in a seven-day study.

as usual. According to Levinson and Hyatt (1970), the optimum for the spore germination lies between 28°C and 38°C which explained the immediate growth of *B. cereus* in the milk when it was placed at 35°C. On the other hand, the growth of vegetative *B. cereus* in milk at the storage temperature of 25°C and 35°C was initiated from bacterial spores. In nature, spore germination is triggered by the presence of specific nutrients as the nutrients germinant will bind to spore-specific protein complexes in a stereospecific manner in the inner membrane (Setlow, 2014). In the present study, although nutrients were present, no spore germination was observed at 8°C which could infer the influence of temperature on spore germination.

There is no evidence for discontinuities in the response of spores to temperature (Levinson and Hyatt, 1970). But, the rate and extent of germination (Vas and Prosz, 1957; O'Connor and Halvorson, 1961; Vary and Halvorson, 1965) and the lag period preceding germination (Keynan and Halvorson, 1962; Vary and Halvorson, 1965) are influenced by the germination temperature. It is a typical magnitude of enzymic reactions (Levinson and Hyatt, 1970). In other words, when the surrounding temperature is near the optimum temperature, the germination process will be triggered when the enzymic reaction is activated.

The growth of vegetative *B. cereus* at 25°C was slower compared to the growth at 35°C when it was not within the optimum growth temperature. Due to the slow growth, the abundance of nutrients present in the milk was slowly consumed by the bacteria (Lafarge et al., 2004; Ray and Bhunia, 2008; de Oliveira et al., 2014) resulting in no production of spores in Days 1 and 2 of storage. In comparison, the vegetative *B. cereus* grew rapidly at 35°C and by Day 2 of storage, there was production of spores when the nutrients depleted. At the same day of storage, the maximum growth of vegetative *B. cereus* was achieved. When compared to the storage at 25°C, the maximum growth was achieved at Day 3. As the incubation time increased, the number of vegetative cells began to decrease gradually due to nutrient depletion. On the other hand, the number of spores began to increase as nutrients reduced and conditions became unfavourable for growth (Setlow, 2014). The trend of the graph shows that the number of vegetative cells decreased while the number of spores increased with incubation time, an inversely proportional relationship which indicates the survival mode of *B. cereus*.

It should be noted that the spore characteristics are greatly influenced by the different genetic of the *B. cereus* strains (Wiencek et al., 1990; Tauveron et al.,

2006; Faille et al., 2007). In addition, Setlow (2006) stated that the spore resistance and dormancy are due to both the unique spore components and spores' unique structure. If there are multiple contaminations of different strains of *B. cereus* spores, it may become a severe issue in decontamination. Notwithstanding, there were other studies which observed *B. cereus* activity such as its toxin production in lower temperature. What was interesting in their finding was, emetic *B. cereus* produces the highest amount of emetic toxin at lower temperatures; 12°C and 15°C (Finlay, 2000). In another related study, *B. cereus* toxin exhibit cytotoxicity at 15°C and 8°C under aerated conditions (Christiansson et al., 1989). In other studies, psychrotrophic *B. cereus* was successfully isolated. They posed threat to public as they were capable of growing under low temperatures and contaminating chilled food (Dufrenne et al., 1994; Choma et al., 2000).

It is widely recognised that *B. cereus* spores are a major concern in food industry. Despite being infamous for its extremely durable endospores, they could also become superdormant spores. Superdormant spores are slow-to-germinate spores and could remain dormant in food by showing no sign of spoilage. They display an extremely long lag period which can last for possibly days, weeks or months (Ghosh and Setlow, 2009; Setlow, 2014). This brought up the concern that superdormant spores could persist in UHT equipment and will remain dormant even after the decontamination process. Although many studies on the model spore former, *B. subtilis*, which represents general conclusion for spores of other *Bacillus* species has been done previously, nothing is known about what is happening during this lag period (Setlow, 2014). However, it was stated that L-amino acids such as L-alanine, L-valine, and L-asparagine trigger germination of *B. subtilis* spores while D-amino acid, on the other hand, is inert (Gould, 1969; Atluri et al., 2005).

The present study simulated the influence of temperature on the germination of spores and growth rate of *B. cereus* in spore-contaminated UHT milk. In the real case scenario, the density of spore contamination in UHT milk may not be high, but the surrounding temperature, especially in the South East Asia region will trigger the germination of the spores and thus, the growth of *B. cereus*. Additionally, UHT milk is stored at room temperatures which increases the probability of *B. cereus* growth in them.

4. Conclusion

In a manner, this study gave us an idea of what might happen in the real scenarios by filling up the data

gap. Extremely resistant surviving spores after sterilization treatment or recontamination could have eventually germinate due to nutrients from milk and ambient temperature that supports their growth. In the matter of surviving spores, routine cleaning for UHT equipment should be done from time to time. Apart from that, effective UHT temperature should be monitored to ensure that no spores could get through the sterilization process. Using the available knowledge, we could also suggest ways to minimize profit loss in companies which produce UHT milk. Additionally, this study also demonstrates milk as a potential medium for *B. cereus* infection.

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