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FULL PAPER

Prevalence of *Bacillus cereus* s.l. in ultra-high temperature chocolate milk from selected milk manufacturers in Malaysia

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Abstract

Bacillus cereus is a major foodborne pathogen of great concern to the dairy industry owing to its resilient spores as well as the adverse effect of its toxins. At present, there is no informational study available to solve or pinpoint the UHT chocolate milk contamination issue in Malaysia. This work aimed to investigate the prevalence and contamination level of *B. cereus* s.l. in UHT chocolate milk and to suggest the appropriate solution for the issue. In the present study, *B. cereus* s.l. prevalence and contamination level in individually packed UHT chocolate milk from processing factories was evaluated. The prevalence and concentration of *B. cereus* s.l. were determined via MPN-PCR (Most Probable Number-Polymerase Chain Reaction) assay. Results showed that 31.11% from 220 of UHT chocolate milk tested contained *Bacillus* spp.; of this *Bacillus* spp. positive samples, 24.30% were also positive for *B. cereus* s.l. with concentration ranging from less than 3 to more than 1100 MPN/mL. Findings from this study highlighted the possibility of UHT chocolate milk as a potential source of *B. cereus* s.l. infection. Therefore, findings emphasized the needs to revise, monitor and improve UHT sterilization process to reduce infection risk. Furthermore, it is also essential to maintain the hygiene to minimize initial microbial load and contamination of UHT chocolate milk, beginning from production to retail.

1. Introduction

Bacillus cereus is a Gram-positive, rod-shaped and spore-forming bacteria which commonly dwells in soil (Jenson and Moir, 2003; Zhou *et al*., 2008). Apart from their natural habitat, they are also widely distributed in the environment (Reyes *et al*., 2007). This bacterium was first defined by Frankland and Frankland (1887) after it was isolated from air in a cowshed (Larsen and Jørgensen, 1997).

Often, this bacterium has been considered as fairly apathogenic; however, *B. cereus* has also been implicated in various infections in man (Kotiranta *et al*., 2000; McDowell *et al*., 2019). Apart from that, *B. cereus* commonly contaminated proteinaceous food such as meat, milk and fish, as well as farinaceous food such as rice, pasta, pastry and noodles (Beecher, 2001; Messelhäusser *et al*., 2014). Foodborne illness related to *B. cereus* appear as two distinct symptoms; diarrhoeal and emetic. Diarrhoeal symptoms often characterised as diarrhoea and abdominal pain, usually eight to 16 hours after consumption of contaminated food; whereas emetic symptoms can be observed as nausea and vomiting, usually one to five hours (Agata *et al*., 2002; Caroll *et al*., 2019). Back in 2013, a massive food poisoning outbreak related to enterotoxigenic *B. cereus* was reported by Schmid *et al*. (2016). The outbreak was due to consumption of contaminated mashed potato dishes containing *B. cereus* loads of 10^5 CFU/g. An outbreak

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involving *B. cereus* was also reported in Italy. The analysis found that basmati rice was contaminated with *B. cereus* and the bacterium was also found in patients' stool (Martinelli *et al*., 2013). Following that, a large outbreak was reported in 55 nurseries in the United Kingdom after the consumption of food prepared by the same company. The microbiological analysis result showed that *B. cereus* was detected in dried haricot beans (Nicholls *et al*., 2016).

B. cereus is a common contaminant in the food industry. It was detected in various foods and food products; among food products that are associated with *B. cereus* that have been discovered in other studies are dried milk products (Reyes *et al*., 2007), RTE cereals (Lee *et al*., 2009; Lesley *et al*., 2013), rice (Sarrías *et al*., 2002; Sandra *et al*., 2012), minced beef (Samapundo *et al*., 2011; Bashir *et al*., 2013; Lesley *et al*., 2017; Food Safety News, 2018), raw milk and pasteurised milk (de Paula Pacheco-Sanchez and de Massaguer, 2007**)**.

Milk is one of many beverages enjoyed by many people from all walks of life due to its nutritional value, with those available in the market are mostly pasteurised and UHT processed. Processed UHT milk has been deemed safe for consumption due to the efficient elimination of microorganisms during UHT processing. In spite of this, as a common contaminant of raw milk (Christiansson *et al*., 1999; Bartoszewicz *et al*., 2008), spores of *B. cereus* can still be detected in the final UHT products (Vyletělová *et al*., 2002; Tortora *et al*. 2016b). Therefore, consuming UHT milk could increase the risk of exposure to foodborne pathogens, specifically *B. cereus* (Bahk *et al*., 2007; Abraha *et al*., 2017).

In Malaysia, UHT milk is a part of the nutritional program with the aim to reduce malnutrition among school children especially those in rural areas. A total of 7613 schools were supplied with UHT milk for the 1Malaysia Milk Programme (PS1M) to benefit 1.53 million children (Bernama, 2012). The programme came to a halt when there was a local outbreak of *B. cereus* in UHT milk which had affected 191 students (Yusof, 2011). To the author's knowledge, there is insufficient study on the microbiological safety on UHT milk, especially flavoured milk which is more favoured by the children. Therefore, the current study was conducted to investigate the prevalence and contamination level of *B. cereus* s.l in UHT milk. The outcome of the study will provide an insight into the UHT process of chocolate milk in Malaysia and identify the possible risk factors. On the whole, data collected from this study will help to contribute towards understanding the risk of *B. cereus* infection through the consumption of UHT chocolate milk.

2. Materials and methods

2.1 Sampling

A total of 214 packages of UHT milk were collected from two manufacturers (A and B – factory names were not to be disclosed due to confidential purposes), in Malaysia were analysed in this study. The samples were transported and immediately analysed upon arrival to the laboratory.

2.2 Most probable number-polymerase chain reaction (MPN-PCR)

Enumeration was performed according to the method described by Lee *et al*. (2009), and Tallent *et al*. (2012) with modifications according to Cappuccino *et al*. (2014) and Sandra *et al*. (2012). Homogenized samples in tryptic soy broth were diluted to 100-fold and 1000-fold for MPN three tubes test and incubated at 37°C for 48 hrs. Turbid MPN tubes were subjected to DNA extraction via boil cell method (Tunung *et al*. 2010). Briefly, one mL of culture was centrifuged at 13, 400 × *g* for 1 min and the pellet was resuspended in 500 µL TE (Tris-EDTA) buffer. The suspension was boiled for 10 min and immediately cooled at -20°C for 10 mins. The suspension was centrifuged again for 1 min at 13, 400 × *g*. The boiled cell lysate was used as template for PCR assay.

Bacillus spp. and *B. cereus* s.l. was detected by PCR assay performed in a 25 µL reaction mixture containing 5 µL of 5× Green GoTaq® Flexi Buffer, 1.5 mM of $MgCl_2$, 0.08 mM of dNTP mix, 0.2 µM of each primer, 1 U/µL *Taq* polymerase (Promega, USA), and 2.5 µL of DNA template. Amplification was performed at 94°C (5 mins) for pre-denaturation, denaturation at 94°C (1 min), annealing at 55°C (1 min), extension at 72°C (1 min) and final extension at 72°C (4 mins). The products were electrophoresed on 1% agarose gel with 1× TBE (Tris-Borate-EDTA) buffer at 100 V for 28 mins. Table 1 shows the list of primer sequences used in this study.

Table 1. Primer sequences used in this study.

Primer	Sequence	Annealing Temperature	Reference	Amplicon Size (bp)
Bacillus spp.	F: 5'-TCACCAAGGCAACGATGCG-3' R: 5'-CGTATTCACCGCGGCATG-3'	55 °C, 1 min	Wu *et al.* (2005)	1114
B. cereus	F: 5'-GAGTTAGAGAACGGTATTTATGCTGC-3' R: 5'-CTACTGCCGCTCCATGAATCC-3'	55 °C, 1 min	Schraft and Griffiths (1995)	411

2.3 Isolation of B. cereus isolates

Turbid MPN tubes were streaked on Mannitol-Yolk-Polymyxin agar and incubated at 37°C for 18 to 24 hrs. Suspected single pink to purple colonies which were surrounded by a ring of dense precipitation of *B. cereus* was picked for further confirmation by PCR.

3. Results and discussion

In this study, *Bacillus* spp. and *B. cereus* s.l. were detected in 31.11% (67/214) and 24.30% (52/214) of the UHT chocolate milk samples, respectively, with MPN distribution ranging from less than 3 to more than 1100 MPN/mL. Majority of the samples were detected with concentration less than 3 MPN/mL; 68.69% and 75.70% for *Bacillus* spp. and *B. cereus* s.l. respectively. For concentration range of 3 to 9.4, 11 to 93, and 120 to 1100 MPN/mL for *Bacillus* spp., the percentage was 19.61%, 5.61% and 0.47% respectively; for *B. cereus* s.l., the percentage were 14.49%, 3.74% and 1.87% respectively. *Bacillus* spp. and *B. cereus* s.l. with concentration more than 1100 MPN/mL account for 6.07% and 4.21% of the samples respectively (Figure 1). Figures 2 and 3 show representative positive samples detected with *Bacillus* spp. and *B. cereus* s.l.

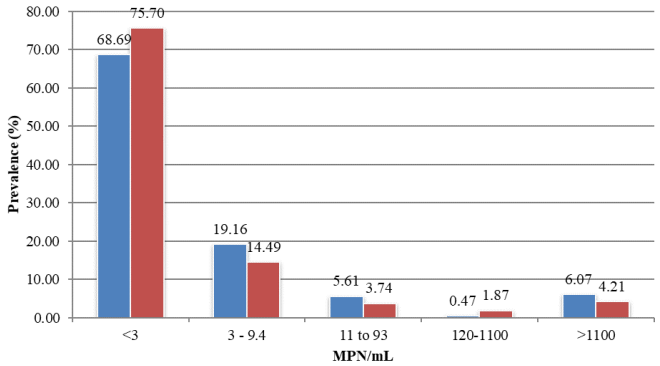

Figure 1. Percentage of contamination level of *Bacillus* spp. (indicated in blue bars) and *B. cereus* (indicated in red bars) in UHT chocolate milk.

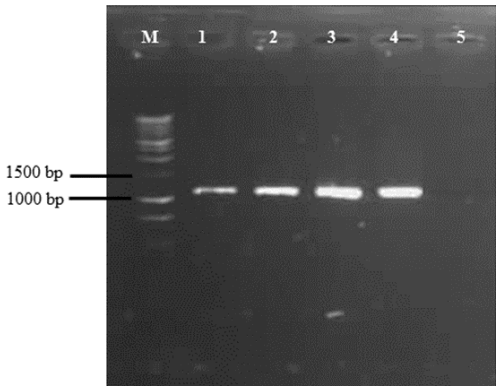

Figure 2. Representative amplification of 16S rRNA gene for the detection of *Bacillus* spp. (1114 bp). Lane M: 1 kb DNA ladder (Promega, USA); lane 1 to 3: representative positive samples at 1114 bp; lane 4: positive control (*B. cereus* ATCC 33019); lane 5: non-*Bacillus* spp. sample.

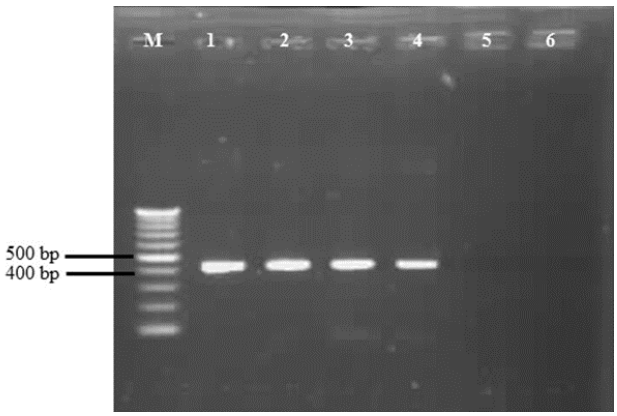

Figure 3. Representative amplification of cereolysin AB gene for the detection of *B. cereus* s.l. (411 bp). Lane M: 100 bp DNA ladder (Promega, USA); lane 1 to lane 3: representative positive samples at 411 bp; lane 4: positive control (*B. cereus* ATCC 33019); lane 5 and lane 6: non-*B. cereus* sample.

The median MPN distribution samples collected in this study is less than 3 MPN/mL, in which it accounts for the majority of the MPN distribution. Thence, it is suggested that majority of the chocolate milk samples were safe for consumption as *B. cereus* s.l. was not detected in any of the samples. However, it should be kept in mind that when samples were 'not detected' with *B. cereus* s.l., it does not mean that the samples were free from the said bacterium. The number of *B. cereus* s.l. was below detected limit, therefore, they were not detected by MPN-PCR assay. Nevertheless, samples with less than 3 MPN/mL could also pose a threat to consumers.

The results of the study were in agreement with Lesley *et al*. (2017) who reported 30% of UHT milk sampled in retail markets in Sarawak, East Malaysia. Vidal-Martins *et al*. (2005) detected *B. cereus* in 11.8% of the 110 samples of UHT milk in their research, wherein the detection is lower compared to the present study. A slightly higher detection was described by Rezende-Lago *et al*. (2007); 58.3% of 120 samples consisting of raw milk, milk powder, UHT milk and pasteurised milk were contaminated with *B. cereus*, but detection was lower in UHT milk (13.3%). The presence of *B. cereus* in UHT milk was also reported in previous studies (Bartoszewicz *et al*., 2008; Ghellai and Moussaboudjemaa, 2013) which are parallel with the present study. Prevalence of *B. cereus* s.l. in this study is higher compared to the prevalence of *B. cereus* in UHT milk mentioned previously in other studies. It can be suggested that it might be due to the high microbial load from raw materials which can lead to the high number of successive spores (Vyletělová *et al*., 2002; Amor *et al*., 2018).

In another related study, Schlegelova *et al*. (2003) reported that 31.0% of dairy foodstuff samples were contaminated with *B. cereus*, showing similar prevalence

to the present study. Another related research stated that *B. cereus* was found in 45.9% of dried milk products (Reyes *et al*., 2007) and analysis by a local study indicated that 78% of 111 RTE cereals were detected with *B. cereus* s.l., showing a higher detection compared to present study (Lee *at al*., 2009). Apart from that, more information on *B. cereus* isolation from various milk and milk products has also been described from the following researches; fresh milk as well as heat-treated milk such as pasteurised milk and UHT milk (Vyletělová *et al*., 2002; Salustino *et al*., 2009; Hassan *et al*., 2010; Fallah *et al*., 2011; Montanhini *et al*., 2013; Kumari and Sarkar, 2016).

UHT processing for milk is a process of continuous heating at temperatures higher than 130°C (generally at 140°C to 150°C) for a holding time of a few seconds (generally 2 to 10 s). Following that, the milk is aseptically packaged to produce a 'commercially sterile' product. Hence, under these conditions, microorganisms that are capable of growing under normal condition of product storage are eventually destroyed (Datta *et al*., 2002; Kjaerulff, 2013). In food industries, UHT processing is the favoured way of sterilising chocolate flavoured milk as it will enhance the flavour of the chocolate without causing bitter aftertaste. What is more, it provides an overall smoothness and favourable taste and at the same time, it extends its shelf-life (Bixler *et al*., 2001; Tiwari and Asgar; 2017). Although the chocolate milk has initially gone through the sterilisation process before being distributed to local retail markets, there were many possible aspects to be considered for *B. cereus* s.l. recontamination on the final products.

Doyle *et al*. (2016) described that the possible reason for contamination of milk occurs during the outdoor grazing period. It was suggested that soil was the main source of contamination for milk by means of cow's teat that came in contact with soil (Christiansson *et al*., 1999; Doyle *et al*., 2016). On that account, the spores eventually came in contact with raw milk during the cows' milking process. Spores in manure may also contribute to some degree of spore in milk, given with high spore content in the soil. In the processing environment, *B. cereus* spores in raw milk will continue to persist after pasteurisation and later on will continue to colonise pipes, storage tanks and filling machines (Jenson and Moir, 2003; Gopal *et al*., 2015). However, most milk manufacturing plants in Malaysia used reconstituted milk for economic reasons. The spores present in raw milk could remain even after the milk was transformed into a powdered form may suggest highly thermo liable spores which are greatly influenced by the genetic group (Sandra *et al*., 2012; Lesley *et al*., 2013).

The spores transfer was suspected to be likely initiated from the raw materials. Commercially produced chocolate milk is usually prepared by mixing raw ingredients such as raw milk or milk powder with cocoa powder, sugar, thickening agent, food stabiliser and preservatives. Selected raw ingredients collected from the factories were subjected to examination for the presence of *B. cereus* s.l. From 32 samples of raw ingredients, 22 (68.6%) samples were detected with *B. cereus* s.l. with MPN distribution ranges from less than 3 MPN/mL to more than 1100 MPN/mL. This indicates that most of the raw materials contained *B. cereus* spores. The spores which might be present in these raw ingredients may successfully be transferred during the milk processing course (Vyletělová *et al*., 2002; Amor *et al*., 2018) and surviving the UHT process, if the spores are incompletely inactivated. Generally, *B. cereus* utilises glucose as its main carbon source (Tallent *et al*., 2012). Apart from that, it is also capable of fermenting sucrose (Aryal, 2019). The high content of sugar in UHT chocolate milk (7.2 g of sucrose per 200 mL of UHT chocolate milk pack) could support the spore germination and growth. In addition, the use of different food ingredients, especially exotic and imported, creates new niches and risk of introducing new unknown heat-stable spore-formers into the production process (Witthuhn *et al*., 2011). According to a study conducted by Witthuhn *et al*. (2011) on the thermal stability of spores, it was concluded that cocoa powder was shown to contain highly heat-resistant spores.

The initial designs of UHT processes focused on the inactivation of thermophilic bacterial spores such as spores of *Geobacillus stearothermophilus* due to their survivability in UHT heating (Witthuhn *et al*., 2011). It was reported that the spores are activated at 121°C and to inactivate the spores requires temperatures well above 121°C (Russell, 1982; Huesca-Espitia *et al*., 2016). However, the introduction of extremely heat-resistant mesophilic spore-former *Bacillus sporothermodurans* (IDF, 2000) had impacted the UHT industry, making a fine line in achieving the inactivation required with the milk quality. In reality, higher temperature will ensure complete inactivation of the spores. Despite the proposed inactivation kinetics, severe chemical changes will be observed, for example, browning, loss vitamins, fouling and gelation, which degrades the quality of milk. What more when the distribution of unknown very heat-stable spores or the evolution of spore-formers such as *B. cereus*, a mesophilic and foodborne pathogen into producing heat-stable spores.

During the production of UHT milk, there is a possibility for the activation of the spores which led to germination. Levinson and Hyatt (1970) reported that the

activation of *B. cereus* spores is within 62°C to 78°C and maximal rate of spore germination was at 64°C to 68°C. The activation temperature is within the range of the holding temperature of most UHT processes which brings the possibility that the spore's germination in a longer indirect heating system, posing a hazard to the design of safe processes.

Apart from their ability to survive harsh environment via endospores, *B. cereus* s.l. is also known to form biofilm which is a huge concern for the food industry. In fact, *B. cereus*' ability to form biofilm has been extensively reported in many researches, colonising surfaces such as stainless steel, glass, plastic, leafy vegetable and polystyrene (Ryu and Beuchat, 2005; Hsueh *et al*., 2006; Houry *et al*., 2009; Elhairy, 2011; Majed *et al*., 2016; Hussain *et al*., 2018). It is possible that during processing, *B. cereus* cells anchor to the surfaces of pipe, storage tanks and filling machines, eventually colonising them (Jenson and Moir, 2003; Gopal *et al*., 2015; Majed *et al*., 2016). They will ultimately produce matrix and form stronger biomasses, with a matrix forming a hydrophobic envelope surrounding the biofilm. The extracellular matrix is primarily comprised of polysaccharides, such as cellulose, proteins or exogenous DNA. The matrix can fix to hard surfaces such as food industry equipment, transport, dispensing and storage surfaces and soil; or to biological structures such as vegetables, meat, bones and fruits. The embedded cells are protected against toxic compounds, due to the matrix's structural role which responsible for durability and persistency of biofilm. Biofilm formation provides many advantages to microbial cells, especially in the food industry setting. For example, physical resistance against desiccation, mechanical resistance against liquid streams in pipelines, and chemical protection against chemicals, antimicrobials and disinfectants used in the food industry (Flemming *et al*., 2016; Galié *et al*., 2018). It can contain spores as well as vegetative cells with spores and result in recontamination during the detachment of these biofilms (Faille *et al*., 2014).

Thenceforth, biofilms are very difficult to eliminate as they exhibit resistant to high temperature and disinfection processes compared to bacteria in the free-living environment. In sync with spores, it gives *B. cereus* high resistance to numerous stress as well as high adhesive capacity on various substrates, which includes stainless steel, a common material used in food processing. Moreover, sporulation occurs at a very high level in biofilms, which makes them difficult to eliminate. It is of great concern when biofilms start to form in areas which are difficult to clean, for example, crevices, valve, gaskets and dead ends (Donlan, 2002; Majed *et al*., 2016).

Contamination of UHT chocolate milk could also take place during the cooling process as milk was passed through the cooling chamber, a potential area for biofilm colonisation. Contamination also causes by the reintroduction of spores during the filling process; when chocolate milk was dispensed into smaller packs. These spores might come from biofilms that have colonised dispenser nozzles and eventually released into previously sterilised chocolate milk. Another possible route of contamination was the chocolate milk could also expose to airborne *B. cereus* spores during the filling process (Actor, 2012). Hence, when optimum temperature is reached for *B. cereus* growth, the spores will germinate and return to their vegetative state (Tortora *et al*., 2016; Majed *et al*., 2016).

It is critical to take note that low numbers of *B. cereus* cells could also cause illness. Lee *et al*. (2009) mentioned in their study on the incident of a low infective dose of *B. cereus* that caused serious food poisoning outbreak in Norway; with a number as low as 10^3 to 10^4 cells/mL, food industries should be a concern (Granum, 1994). A total of 4.21% of UHT milk samples from this study contained more than 1100 MPN/mL of *B. cereus*. Considering the fact that 10^3 cells/mL could lead to infection, this UHT milk could pose risk to consumers. In addition to that, milk is a good medium to support the germination of *B. cereus* due to its nutritious content (Ray and Bhunia, 2008). As such milk is consumed more by infant and children for its nutrients and who bear the highest foodborne disease burden (Kirk *et al*., 2015), it is, therefore, necessary to monitor the microbiological safety of milk.

4. Conclusion

Detection of *B. cereus* s.l. and other foodborne pathogens is vital for the safety of food products. Findings from this study highlighted the possibility of UHT chocolate milk as a potential source of *B. cereus* s.l. infection. In this manner, it is of significant important to revise, monitor and improve UHT sterilization and packaging processes in order to reduce infection risk. From food safety point of view, thorough and continuous monitoring of *B. cereus* s.l. during UHT milk production process is important in order to address *B. cereus* s.l. infection issue as UHT milk is a potential medium to acquire its infection. Furthermore, it is also imperative to supervise and maintain the hygienic level to minimize initial microbial load and contamination, beginning from production to table in order to safeguard consumers along with public health.

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Conflict of Interest

The authors declare no conflict of interest.

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