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Detection of *Bacillus cereus* in formula milk and ultra high temperature (UHT) treated milk products

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Abstract

Ultra high temperature (UHT) treated milk products and formula milk are known to be frequently contaminated with *Bacillus cereus*. Presence of *B. cereus* in these milk products is of particular concern considering the majority of consumers are infants and children. Possible sources of contamination are contaminated raw milk, cross-contamination during processing, under-processing and mishandling of milk products. This study was conducted to detect the presence of *B. cereus* in both formula milk (n=12) and UHT milk (n=20) sold in selected retail markets. The approach consisted of enumerating by MPN/g followed by PCR assay aimed at detecting *gyrB* gene in *B. cereus,* that encode for the subunit B protein of DNA gyrase (topoisomerase type II). Contamination level of *B. cereus* in both types of samples examined ranged from < 3 to > 1100 MPN/g. The contamination level of *B. cereus* was found to be highest in full cream UHT milk (> 1100 MPN/g) and formula milk (> 1100 MPN/g). The PCR analysis showed that 41.7% (5/12) formula milk and 30% (6/20) UHT milk samples were detected with *B. cereus*, respectively. This is the first report of such study demonstrating the presence of *B. cereus* in formula milk from Malaysia. Therefore, constant surveillance of these milk products would reduce the potential risk of *B. cereus*-linked outbreaks.

Introduction

B. cereus is a Gram positive, facultative anaerobe, rod-shaped bacterium, a common soil-dweller and has the capability to form endospores which allows it to survive in extreme environmental conditions (Montanhini and Bersot, 2013). Due to their resistant endospores, the pathogen can thrive in various food processing procedures such as drying and heat treatment (Rosenquist *et al.*, 2005; Tunio *et al.*, 2013). *B. cereus* has been reported in various foods such as dairy products, rice, vegetables and meat (Lee *et al.*, 2010; Tunio *et al.*, 2013). It is responsible for two different types of gastrointestinal disorders: emetic syndrome caused by ingestion of a preformed toxin in the food, and diarrheal syndrome, caused by a different toxin that can be formed in the food but also in the small intestine (Reyes *et al.*, 2007). Fatality linked to *B. cereus* had also been reported due to the consumption of contaminated pasta (Dierick *et al.*, 2005).

Based on a report by European Food Safety Authority (EFSA) in 2005, 1 to 33% of food borne poisoning was attributed to B. cereus (Sandra *et al.*, 2012). An increment of 122.2% in food poisonings cases caused by *B. cereus* in Europe were reported to the EFSA in the year 2011 (Messelhäusser *et al.*, 2014). Food poisoning cases associated with milk-based products have been reported and as high as 85% of enterotoxigenic *B. cereus* have been isolated from milk and milk products (Sadek *et al.*, 2006). Occurrences of *B. cereus* in milk products are especially important concern in the baby formula industry. Infants are more susceptible to food borne infections due to under-developed immune system and absence of competing microorganisms in gut microflora. Moreover, infant and toddler milk powder often contain raw ingredients from various sources that are rich in nutrients. When reconstituted and left at ambient temperatures for a long periods, these milk products will become a suitable medium for proliferation and enterotoxin production of *B. cereus* (Tunio *et al.*, 2013). Hence, frequent exposure of infants and toddlers to these milk products increases the risk of contracting food borne illness. Recall of infant feeding products has been reported upon development of gastrointestinal disorders in infants which may be caused by microbial contamination

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(Walker, 2010; Samakow, 2012). In the year 2011, a recall for Australian UHT skim milk had been reported which was due to microbial spoilage (Food Standards Australia New Zealand (FSANZ), 2014). Incidences of *B. cereus* in processed milk products have also been reported (Messelhäusser *et al.*, 2014). *B. cereus* spores are known to survive pasteurization and had been isolated from ultra high temperature (UHT) treated milk (Ubong *et al.*, 2013).

Local studies on *B. cereus* in cooked foods, rice noodles, wet wheat noodles, dry wheat noodles, spices, grains, legumes and legume products, ready to eat cereals, chocolate, honey, milk, ready-to-eat cooked rice, imported raw rice have been reported (Rusul and Yaacob, 1995; Lee *et al.*, 2009; Sandra *et al*., 2012; Lesley *et al*., 2013; Ubong *et al*., 2013). Previously, a local outbreak associated with *B. cereus* in contaminated UHT milk was reported which had affected 191 students (Yusof, 2011). Therefore, the aims of this study were to determine the presence and level of *B. cereus* in formula milk and UHT milk from retail outlets in Kuching, Sarawak.

Materials and Methods

Sampling and samples preparation

A total of 32 samples of formula milk (infant formula and follow-up) (n = 12) and UHT milk (full cream and low fat) (n = 20) were purchased directly from various retail markets between September and December 2014. The samples were transported in ice box to the laboratory and processed immediately. Ten mL of the UHT milk were enriched in 90 mL of Tryptic Soy broth (TSB; Merck KGaA, Germany) and incubated at 37°C for 24 hours. As for formula milk, 25 g of the formula milk powder were reconstituted with 225 mL of TSB and incubated at 37°C for 24 hours. Each milk sample was prepared in triplicates and followed by enumeration method.

Enumeration of B. cereus *from milk samples*

Enumeration of *B. cereus* was performed using 3 x 3 most probable number (MPN) method. Briefly, ten–fold serial dilutions from 10^{-1} – 10^{-3} were made with TSB by inoculating 1 mL of the enriched sample into a Falcon tube containing 9 mL of TSB. Subsequent dilutions were made up to 10-3 in triplicates. The tubes were incubated at 37°C for 18 – 24 h until the turbidity was observed. Selected turbid, positive tubes were subjected to biomolecular analysis to determine the presence of B. cereus in the milk samples examined.

Biomolecular analysis

DNA templates were prepared from 1 mL of the turbid, positive MPN tubes boil-cell method as described by Sandra *et al.* (2012). PCR was performed by using a thermocycler (Swift™ MiniPro Thermal Cycler, ESCO, US). A 20 µL PCR reaction mixture which contain 5.0 µL of 5× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of dNTP mix, 1.0 µM of each primers, 0.2 U/µL Taq polymerase and 2.0 µL of DNA template was prepared. The presence of B. cereus was screened by PCR using the primer pair of BCJH-F (TCATGAAGAGCCTGTGTACG) and BCJH-1R (CGACGTGTCAATTCACGCGC) (Bio Basic Canada Inc., Canada) for specific amplification of a 475 bp fragment of *gyrB* gene (Sandra *et al*., 2012). The reference *B. cereus* strain ATCC 33019 was included as a positive control. The PCR products were analyzed by 1.5% agarose gel electrophoresis. Five µL of the amplified products were loaded onto the agarose gel buffered in 1× TBE buffer and subjected to electrophoresis for 60 min at 90 V. The gel was stained with ethidium bromide (0.5 µg/ml) (Sigma, US) and visualized by UltraSlim UV Transilluminator (Maestrogen, US).

Results and Discussion

Among 12 samples of formula milk examined, five samples were *B. cereus*-positive accounted for 41.7% of total tested products. The contamination rate of *B. cereus* was the highest in young-child formula accounted for 33.3% (n = 10) of the total samples. Of all the follow-up formula products tested, only one sample was detected with *B. cereus*. However, none of the infant formula samples were contaminated by *B. cereus.* Contamination level of *B. cereus* in the samples tested ranged from < 3 to > 1100 MPN/g.

Out of the 20 samples of UHT milk, 30% (n = 6) of the samples examined were contaminated by *B. cereus*. Among these, 15% of full cream milk (n = 3) was found to be most contaminated, followed by 10% of low fat milk (n = 2) and whole milk 5% (n = 1). Both skimmed milk and fresh milk were not detected with *B. cereus*. Enumeration of *B. cereus* in the milk samples showed that the concentration was in the range of < 3 to > 1100 MPN/mL. Occurrence of *B. cereus* in various types of formula milk and UHT milk is summarized in Table 1.

In this study, the results revealed that the formula milk powders (41.7%) and UHT milks (30%) sold in local retail markets were contaminated by *B. cereus.* According to FSANZ (2004), contamination rate of 9 - 48% was reported in milk and dairy products which is in agreement with the findings of this study. The

Table 1. Occurrence of *B. cereus* in different types of formula milk and UHT milk

Sample	Types of sample	No. of samples assessed	*B. cereus* positive (%)
Formula Milk	Infant formula	3	0 (0)
	Follow-up formula	3	1 (8.3)
	Young-child formula	6	4 (33.3)
UHT Milk	Whole milk	3	1 (5)
	Full cream milk	8	3 (15)
	Low fat milk	6	2 (10)
	Skimmed milk	2	0 (0)
	Fresh milk	1	0 (0)

presence of *B. cereus* in the dehydrated formula milk products is supported by Tunio *et al.* (2013). They reported that five powdered food products including infant formula milks and powdered protein-based shakes purchased from retail stores in Pakistan were contaminated by *B. cereus*. Despite the different categories of formulated milk products, similar results was also reported by Rahimi *et al.* (2013) whereby 42% (84/200) of the infant foods examined in Iran were found to be contaminated by *B. cereus*. Soleimaninanadegani (2013) stated that the effective dose of *B. cereus* consumed to develop the symptoms of illness is 10^5 to 10^6 cells or spores/g. The maximum contamination level of *B. cereus* in formula milk in this study was more than 1100 MPN/g of sample. High number of *B. cereus* found in the formula milk in this study indicates that the products may be unsatisfactory for consumption. Considering the majority of target consumers are infants and young children, lower number of *B. cereus* in a range of 10^2 to 10^5 CFU/g of food may also lead to food intoxication (Environment Canada, 2013). According to the guideline levels for determining the microbiological quality of ready-to-eat foods, levels of *B. cereus* <1-2 CFU/g and $\geq 10^4$ CFU/g are considered satisfactorily or potentially hazardous, respectively (FSANZ, 2001). Likewise, Di Pinto *et al.* (2013) reported that 5 out of 11 powdered infant formula samples showed positive result for *B. cereus* but the contamination level was below the infectious dose. *B. cereus* was among the primary microorganisms associated with infant formula contamination as reported by FAO/WHO Expert Consultations previously (Wang *et al.*, 2009). Hence, deeper consideration should be given to infants and young children when assessing the risks of formula milk products because they have weaker of immunity compared to healthy adults (Tunio *et al.*, 2013).

UHT milk is a type of processed milk that is heat-treated at a temperature of 138 °C for a short period of time of around 1-2 second(s) and aseptically packaged (Neumann *et al.*, 2010). Although heat treatment and stringent quality control measures were taken in the production stages, *B. cereus* was still found in UHT milk such as observed in this study. According to the results, UHT milk showed a relatively high percentage with 30% (n = 6) of contamination which is in agreement with the results of Ubong *et al*. (2013). Ubong *et al.* (2013) reported that 13.3% (4/30) of UHT chocolate-flavored milk samples purchased from retail markets in Malaysia was contaminated by *B. cereus* with contamination level ranged from < 3 to 11 MPN/mL. The findings of the present study showed a higher number of *B. cereus* in UHT milk samples, reaching up to > 1100 MPN/mL. The microbiological limit of *B. cereus* in manufactured milk products is less than 10^3 *B. cereus*/g (FSANZ, 2004). Hence, the current findings indicate that the number of *B. cereus* in UHT milk samples exceed the standard microbiological limit and may not be safe for consumption. Even though the milk products were contaminated with *B. cereus*, it may not necessary lead to food poisonings as this bacterium requires desired conditions to produce toxins or the concentration of this organism is insufficient to cause the symptoms (Sandra *et al*., 2012). However, consumption of milk products with B. cereus levels exceeding the standard limits may still be a potential risk to the consumers particularly among the susceptible groups which are immuno-compromised, elders, pregnant mothers and infants.

Conclusion

This study provides an overview of the presence of *B. cereus* in formula milk and UHT milk. The study highlighted the importance of milk as the transmission mode of pathogen to the public and raise a public health concern. Considering that infants and children are the major consumers of formula milk and UHT milk, detection of *B. cereus* in these products is crucial as they are more susceptible to food borne illnesses. The presence of *B. cereus* in milk especially infant formula can be an alarming threat as reconstitution of the dried milk powder may promote germination of *B. cereus* spores and production of

toxin which causing diseases. It is suggested that further studies on other potential pathogenic *Bacillus* spp. that could be involved in foodborne poisoning are needed, particularly in milk and milk products, to increase the knowledge on pathogenic *Bacillus* spp. Besides, it is to implement effective preventive measures to curb related foodborne outbreaks in the future. Additionally, constant surveillance of these milk products would reduce the potential risk of *B. cereus*-linked outbreaks.

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