

**Growth and enterotoxin production of *Bacillus cereus* in cow, goat, and sheep milk**Lenka Necidová<sup>1</sup>, Šárka Bursová<sup>1</sup>, Alena Skočková<sup>1</sup>, Bohdana Janštová<sup>2</sup>,  
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**Abstract**

The aim of this study was to compare *Bacillus cereus* growth rates and diarrhoeal enterotoxin production in raw and pasteurized goat, sheep, and cow milk in terms of storage conditions. Milk samples were inoculated with *B. cereus* (CCM 2010), which produces diarrhoeal enterotoxins. Enterotoxin production was tested by ELISA (Enzyme-Linked Immunosorbent Assay), and the count of *B. cereus* was determined by the plate method. With raw cow milk, *B. cereus* growth and enterotoxin production can be completely suppressed; in raw goat and sheep milk, enterotoxin was produced at 22 °C. In pasteurized cow, goat, and sheep milk, the *B. cereus* count increased under all storage conditions, with more rapid growth being observed at 15 °C (sheep milk) and 22 °C (cow and goat milk). Enterotoxin presence was detected at 15 °C and 22 °C, and with pasteurized cow milk also at 8 °C. Our model experiments have determined that *B. cereus* multiplication and subsequent enterotoxin production depend on storage temperature and milk type.

*Food safety, diarrhoeal enterotoxins, raw milk, pasteurized milk*

The high level of nutrients in milk makes it an especially suitable growth medium for bacteria. In fact, these microorganisms can achieve high population densities following contamination during milk processing. In particular, contamination of milk by members of the *Bacillus cereus* group is of significance, not only because of their spoilage capability, but also because of their potential to cause human diseases (Janštová et al. 2006; Bartoszewicz et al. 2008). Svensson et al. (2007) in their study have assumed that the mesophilic isolates of *B. cereus* from the farm, silo tanks, and pasteurized milk are often high producers of enterotoxin. Major sources of *B. cereus* in pasteurized milk are spores in raw milk (Lin et al. 1998).

*Bacillus cereus* can grow in most foods at a pH above 4.5 and temperatures above 4 °C (van-Netten et al. 1990; Granum 2005). Storage temperature is an important factor in order to keep the *B. cereus* number low. At a dairy, milk is generally kept at 4 °C, which helps to assure longevity. During distribution, however, temperatures of up to 8 °C are common. Furthermore, the consumer often exposes milk to higher temperatures. The increase in numbers of *B. cereus* is tremendous when the storage temperature is elevated by just 2 °C, from 6 °C to 8 °C (Andersson et al. 1995).

There are two different types of *B. cereus* food poisoning. The first type caused by an emetic toxin results in vomiting; the second type caused by one of three different enterotoxins induces diarrhoea (Granum 2001; Svensson et al. 2004). Diarrhoeal syndrome incubation period is 8–16 h, and duration of illness is 12–24 h. The symptoms are abdominal pain, watery diarrhoea, sometimes with nausea (Granum 1994, 2000, 2001). The total infective dose for diarrhoeal syndrome seems to vary between about  $10^5$  and  $10^7$  viable cells or spores. Thus, any food containing more than  $10^3$  *B. cereus*·g<sup>-1</sup> can not be considered completely safe for consumption (Granum et al. 1997). The variation in

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the infective dose may be due to the ability of different strains to produce enterotoxin and to different susceptibilities of individuals (Granum et al. 2000). The Czech Standard ČSN 56 9609 (2005) sets the maximum limit values of  $10^5$  CFU·ml<sup>-1</sup> (log 5.00 CFU·ml<sup>-1</sup>) of *B. cereus* for foods not intended for direct consumption and  $10^4$  CFU·ml<sup>-1</sup> (log 4.00 CFU·ml<sup>-1</sup>) for foods intended for direct consumption. The diarrhoeal toxins may be produced by psychrotrophic strains at temperatures down to 4 °C (Granum et al. 2000).

The aim of this study was to compare the growth rates of *B. cereus* and diarrhoeal enterotoxin production in raw and pasteurized goat, sheep, and cow milk in terms of varying storage conditions.

### Materials and Methods

*Bacillus cereus* strain CCM 2010 producing enterotoxins was obtained from the Czech Collection of Microorganisms (Brno, Czech Republic). Cow, goat and sheep milk singular samples were investigated, both raw and pasteurized. Raw cow milk samples were obtained from a milk vending machine, and goat and sheep milk samples came from farms. Pasteurization was carried out at 75 °C for 20 s. Milk samples negative for *B. cereus* were inoculated with log 3.00–4.74 CFU·ml<sup>-1</sup> of *B. cereus* CCM 2010. Inoculated milk samples were incubated at 8 °C, 15 °C, and 22 °C for seven days to simulate inappropriate transport and storage conditions. The cultures were sampled on days 0–4 and 7. During the incubation, pH of the model samples was measured. Enumeration of *B. cereus* was performed using the Manitol Yolk Polymyxin B agar (Oxoid, Basingstoke, GB) plate count method in accordance with ČSN EN ISO 7932 (2005), and the plates were incubated aerobically at 30 °C for 24 h. To detect enterotoxins, the Diarrhoeal Enterotoxin Visual ImmunoAssay (TECRA, Roseville New South Wales, Australia) was used following the manufacturer's instructions.

### Results

Our experiments revealed variation in *B. cereus* counts during the culture period and in the time to enterotoxin production, depending on the storage conditions and type of milk.

In raw cow milk inoculated with *B. cereus* at log 3.70 CFU·ml<sup>-1</sup>, stored at 8 °C, 15 °C, and 22 °C, the bacterium count decreased to < log 0.69 CFU·ml<sup>-1</sup> after 24 h and remained so during the entire experiment. No enterotoxin production was detected (Table 1). As *B. cereus* was not detected in raw cow milk at a pH of 6.59, where pasteurized milk was positive for *B. cereus* and enterotoxin, we conclude that *B. cereus* growth is influenced not by pH count but by competitive microbiota not present in pasteurized milk.

Table 1. Time to the first detection of *Bacillus cereus* enterotoxins in different types of milk

Type of milk	Raw milk			Pasteurized milk		
	Storage temperature					
	8 °C	15 °C	22 °C	8 °C	15 °C	22 °C
Day of storage						
Cow milk	-	-	-	7	2	1
Goat milk	-	-	1	-	2	1
Sheep milk	-	-	4	-	4	1

Fig. 1 records the increase in the *B. cereus* count in pasteurized cow milk. At both 15 °C and 22 °C, the recommended limit of log 4.00 CFU·ml<sup>-1</sup> (ČSN 569609, 2008) was exceeded prior to 24 h of storage. Storage temperature significantly influences the growth of *B. cereus* and enterotoxin production in pasteurized cow milk (Table 1).

In raw goat milk stored specifically at 15 °C and 22 °C, the recommended limit of log 5.00 CFU·ml<sup>-1</sup> (ČSN 569609, 2008) was exceeded prior to 24 h of storage. No enterotoxin production was detected in raw goat milk stored at 8 °C and 15 °C, although enterotoxin was detected after 24 h in milk stored at 22 °C (Table 1). Fig. 2 shows a higher overall

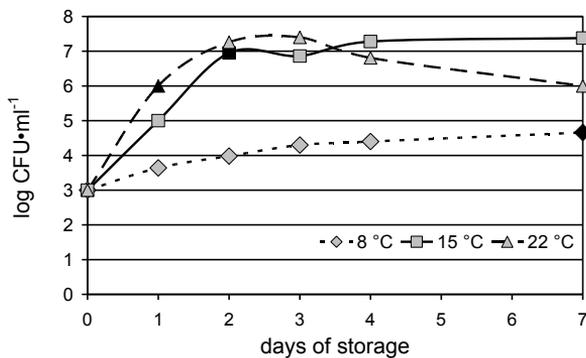


Fig. 1. *Bacillus cereus* growth in pasteurized cow milk

Note: The black shapes indicate the first detection of enterotoxins

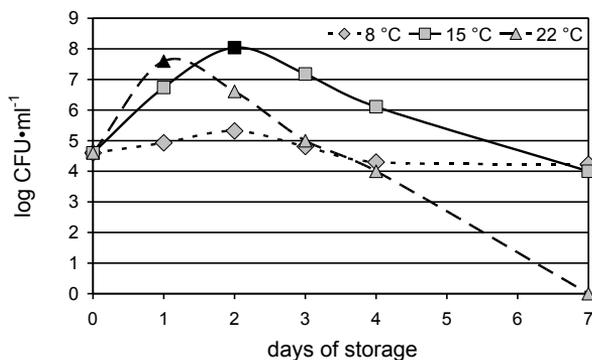


Fig. 2. *Bacillus cereus* growth in pasteurized goat milk

Note: The black shapes indicate the first detection of enterotoxins

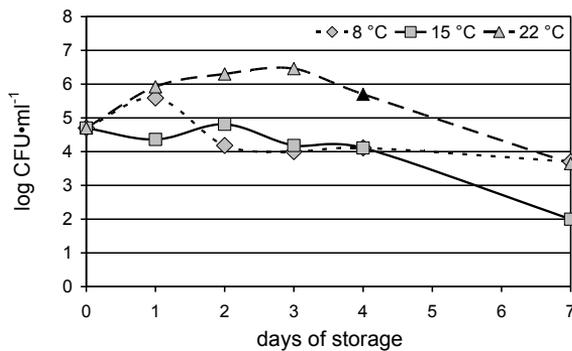


Fig. 3. *Bacillus cereus* growth in raw sheep milk

Note: The black shapes indicate the first detection of enterotoxins

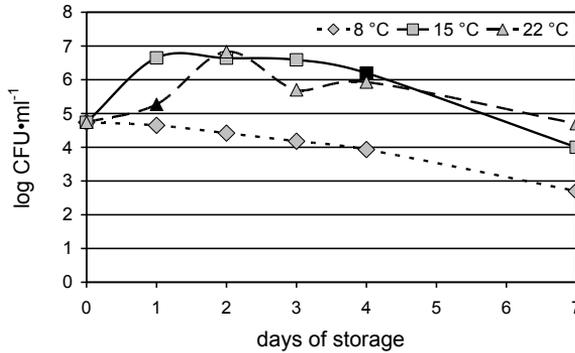


Fig. 4. *Bacillus cereus* growth in pasteurized sheep milk

Note: The black shapes indicate the first detection of enterotoxins

*B. cereus* count at 15 °C and at 22 °C. Presence of enterotoxin was detected after 2 days in pasteurized goat milk stored at 15 °C and within 24 h in pasteurized goat milk stored at 22 °C (Table 1).

Figs 3 and 4 show *B. cereus* growth in both raw and pasteurized sheep milk. Table 1 indicates detection of enterotoxin in raw sheep milk after 4 days of storage at 22 °C. At 15 °C, enterotoxin was detected in pasteurized sheep milk after 4 days of storage, although at 22 °C, enterotoxin was detected prior to 24 h of storage.

## Discussion

According to Christiansson (1993), the number of *B. cereus* present in food must be at least 10<sup>6</sup>·g<sup>-1</sup> or ml<sup>-1</sup>, and sufficiently high amounts of enterotoxin must be formed, to survive the pH of stomach and the proteolytic enzymes of the duodenum (Granum 1994; Granum et al. 2000). Granum (2005) has also reported that the growth of *B. cereus* is prevented with a pH below 4.5. This is confirmed in part by our results showing a rapid decrease in pH below 4.5 in raw cow milk stored at higher temperatures, with *B. cereus* growth being inhibited.

In contrast to raw cow milk, *B. cereus* multiplied in raw goat and sheep milk at 8 °C within the first 24 h, with its count only showing a slight downward trend during the entire storage period. Although the limit of log 5.00 CFU·ml<sup>-1</sup> was exceeded in isolated cases, toxin production was not detected. In raw goat milk at higher temperatures, natural competitive microbiota and decreased pH quickly inhibited *B. cereus* growth. The initial sharp rise of *B. cereus* counts at 22 °C was high enough to produce enterotoxins. The increase in *B. cereus* count in sheep milk was considerably higher at 22 °C than at 15 °C, but the opposite was true for both goat and cow milk. A possible explanation for this phenomenon could be a different composition of sheep milk natural microbiota and a slower pH drop that might have been influenced by *B. cereus* proteolytic activity. Our results indicate that unlike cow milk, raw goat milk and particularly sheep milk are better substrates for multiplication of *B. cereus* where enterotoxins may be produced at higher storage temperatures.

Previous studies also noted that *B. cereus* growth in raw cow milk is very low and that higher numbers of *B. cereus* are detected in pasteurized and heat-treated milk (Svensson et al. 2004; Adams et al. 2008). Pasteurized milk was a good substrate for enterotoxin production. In pasteurized sheep and goat milk stored at 8 °C, no enterotoxin production was detected, even when the *B. cereus* count increased above the recommended limit of log

4.00 CFU·ml<sup>-1</sup>. Although Andersson et al. (1995) have reported that there are strains of *B. cereus* that may cause food poisoning with an infective dose as low as 10<sup>3</sup>–10<sup>4</sup> bacteria/gram, our results are rather consistent with the conclusion of Langeveld et al. (1996) that there is no evidence that *B. cereus* concentrations less than 10<sup>5</sup>·ml<sup>-1</sup> cause intoxication. In pasteurized cow's milk stored at 8 °C, the presence of diarrhoeal enterotoxin was detected after 7 days. At higher storage temperatures, *B. cereus* counts rose sharply in pasteurized milk of all types, considerably exceeding the limit of log 4.00 CFU·ml<sup>-1</sup>, which was associated with enterotoxin production. Therefore, we conclude that maintaining the cold chain at 8 °C or less, in accordance with the legislation in force (Decree No. 77/2003), and consuming milk within seven days minimizes the risk of *B. cereus* diarrhoeal enterotoxin. According to Andersson et al. (1995), with a low number of spores in the products and proper cooling (at least not exceeding the temperature of 6 °C), *B. cereus* food poisoning through milk and milk products can be avoided, as very few strains grow at 6 °C and below.

Our model experiment results have determined that *B. cereus* multiplication and subsequent enterotoxin production depend on storage temperature and type of milk. Pasteurized milk is a more suitable substrate for *B. cereus* multiplication and enterotoxin production than raw milk. With raw cow milk in contrast with sheep and goat milk, *B. cereus* growth and enterotoxin production are more markedly suppressed by competitive microbiota. Nevertheless, this finding should not be interpreted as a reason to encourage consumption of raw, unpasteurized milk. Finally, our study results reinforce the importance of maintaining the cold chain (at a safe temperature under 8 °C) from production through retail sale to insure safety of milk and dairy products.

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#### References

- Adams MR, Moss MO 2008: *Bacillus cereus* and other *Bacillus* species. In: Adams MR, Moss MO (Eds): Food Microbiology, 3<sup>rd</sup> edn. Royal Society of Chemistry, Cambridge, pp. 185-190
- Andersson A, Ronner U, Granum PE 1995: What problems does the food industry have with the spore-forming pathogens *Bacillus cereus* and *Clostridium perfringens*? Int J Food Microbiol **28**: 145-155
- Bartoszewicz M, Hansen BM, Swiecicka I 2008: The members of the *Bacillus cereus* group are commonly present contaminants of fresh and heat-treated milk. Food Microbiol **25**: 588-596
- Christiansson A 1993: Enterotoxin production in milk by *Bacillus cereus*: a comparison of methods for toxin detection. Neth Milk Dairy **47**: 79-87
- ČSN 569609 2008: Guide to good hygiene and manufacturing practise – Microbiological criteria for foods. Principles for establishment and application (in Czech), 40 p.
- ČSN EN ISO 7932 2005: Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of presumptive *Bacillus cereus* – Colony-count technique at 30 °C (in Czech), 20 p.
- Decree No 77 of 6 March 2003 laying down the requirements for milk, dairy products, ice creams, and edible fats and oils. Code, 2003, part 32, pp. 2488-2516
- Granum PE 1994: *Bacillus cereus* and its toxins. J Appl Bacteriol Suppl **76**: 61S-66S
- Granum PE, Lund T 1997: *Bacillus cereus* and its food poisoning toxins. FEMS Microbiol Lett **157**: 223-228
- Granum PE, Baird-Parker TC 2000: *Bacillus* spp. In: Lund B, Baird-Parker TC, Gould GW (Eds): The microbiological safety and quality of food. Aspen Publishers MD, USA, pp. 1029-1039
- Granum PE 2001: *Bacillus cereus*. In: Doyle MP, Beuchat LR, Montville (Eds): Food Microbiology, 2<sup>nd</sup> edn. Fundamentals and Frontiers. ASM Press, Washington DC, pp. 373-381
- Granum PE 2005: *Bacillus cereus*. In: Fratamico PM, Bhunia AK, Smith JL (Eds): Foodborne Pathogens: Microbiology and Molecular Biology. Caister Academic Press, Norfolk, pp. 409-419
- Janštová B, Dračková M, Vorlová L 2006: Effect of *Bacillus cereus* enzymes on the milk quality following ultra high temperature processing. Acta Vet Brno **75**: 601-609
- Langeveld LPM, van Spronsen WA, van Beresteijn ECH, Notermans SHV 1996: Consumption by healthy adults of pasteurized milk with a high concentration of *Bacillus cereus*: A double-blind study. J Food Protect **59**: 723-726

- Lin S, Schraft H, Odumeru JA, Griffiths MW 1998: Identification of contamination sources of *Bacillus cereus* in pasteurized milk. *Int J Food Microbiol* **43**: 159-171
- Svensson B, Ekelund K, Ogura H, Christiansson A 2004: Characterisation of *Bacillus cereus* isolated from milk silo tanks at eight different dairy plants. *Int Dairy J* **14**: 17-27
- Svensson B, Monthán A, Guinebretière MH, Nguyen-Thé C, Christiansson A 2007: Toxin production potential and the detection of toxin genes among strains of the *Bacillus cereus* group isolated along the dairy production chain. *Int Dairy J* **17**: 1201-1208
- van Netten P, van de Moosdijk A, van de Hoensel P, Mossel DA, Perales I 1990: Psychrotrophic strains of *Bacillus cereus* producing enterotoxin. *J Appl Bacteriol* **69**: 73-79