Growth Dynamics of *Bacillus cereus* and Shelf-life of Pasteurised Milk

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Abstract

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Growth dynamics of *Bacillus cereus* in pasteurised milk was examined in storage tests performed at 5, 7, 9, 11, and 13°C. The contents of *B. cereus* in pasteurised milk ranged from the absence in 4 ml of milk to 2.3 cfu/ml. The initial total plate counts varied from 1.1×10^4 to 3.0×10^4 cfu/ml (n = 15). Growth curves of *Bacillus cereus* showed that strains naturally present in pasteurised milk grew well at 5 and 7°C. A square root model was used for the growth rate analysis in relation to the storage temperature of milk ($\sqrt{\mu} = 0.026\ (T - T_{min})$; $R^2 = 0.93$). Lag-time of *B. cereus* was described by a modified Arrhenius-type equation ($\lambda = -45.667 + 1035.3/T$; $R^2 = 0.94$). The comparison of the time prediction calculated for *B. cereus* to reach the density of 10^4 cfu/ml in pasteurised milk and that for the total plate counts to reach levels of 5.0×10^4 cfu/ml proved that these plate counts were reached in all tests earlier than the level of 10^4 cfu/ml could be reached by *B. cereus*. It is thus concluded that pasteurised milk is spoiled by the growth of saprophytic psychrotrophic bacteria before *B. cereus* can produce hazardous levels of its enterotoxin.

Keywords: pasteurised milk; *B. cereus*; shelf-life

The gradual transition of food retailing patterns from small stores to large supermarkets has been associated with an extension of the shelf life of the perishable foods. This applies also to pasteurised milk. The shelf life of pasteurised milk sold in Slovakia has been extended from 3 days to 5–7 days during the last ten years. To achieve a longer shelf life of raw milk, it is inevitable to keep it at the temperatures below +5°C before and also after its production, during transportation, and in retail storage.

In Slovakia, processed pasteurised milk has to comply with three microbiological requirements of the EU directive EC Council Directive 92/46/EEC:
(1) the total plate count (at 30°C) must not exceed 3×10^4 cfu/ml in fresh pasteurised milk,
(2) the total plate count must not exceed 5×10^4 cfu per ml in pasteurised milk incubated at 6°C for 5 days,
(3) coliforms must not be found in 1 ml.

No limit for *Bacillus cereus* is listed in this directive mentioned.

Generally, almost all viable bacteria present in milk are killed by a regular pasteurisation. However, approximately 0.1% of the natural microflora in pasteurised milk may survive the pasteurisation temperature and promote spoilage of the product. In Slovakia, two pasteurisation regimes are used by the producers, i.e. heat treatment at 74°C for 15 to 40 s, or at 85°C for a few (approx. four) seconds. *Bacillus* spp. and *Corynebacterium* spp. are usually found in addition to either surviving or contaminating bacteria although thermoresistant micrococci and streptococci may also be found occasionally. The coryneforms, micrococci, and streptococci are usually unable to grow in pasteurised dairy products stored at temperatures below 6°C (Muir 1996).

Bacillus cereus is the most important of the spore-forming microorganisms because its spores are ubiquitous in raw milk, survive the pasteurisation process, and produce different enterotoxins which may cause food poisoning of the diarrhea or emetic types (Granum *et al*. 1993; Notermans & Tatini 1993). Moreover, many of the strains can grow at 4–6°C. Recently, a new psychrotolerant species, *B. weihenstephanensis*, was detected by Lechner *et al.* (1998) and Mayr *et al.* (1999).

Heat resistance of *B. cereus* spores varies with different strains. The following D_{95}-values of different *B. cereus* strains were found: 8.2–8.4 min by Laurent *et al.* (1999), 4.4–4.6 min by Gonzáles *et al.* (1999), 0.9–11 min by Fernándes *et al.* (2001) at pH = 7, and 0.80 to 1.68 min at pH = 6.5 adjusted by organic acids by Leguerinel and Mafart (2001). D-values of *B. cereus* spores published by Muir (1996) were at 121°C in the range of 2–4 s.

Spores of *B. cereus* are very hydrophobic (Doyle *et al.* 1984) and can adhere to the surfaces of stainless steel. The attached spores may subsequently germinate, multiply, take part in forming biofilm and then resporulate (Andersson *et al.* 1995; Te Giffel *et al.* 1997; Aantrekker *et al.* 2000; Peng *et al.* 2001).

B. cereus outbreaks were associated mostly with the ingestion of catered meals (Todd 1995; Mossel *et al.* 1999). Only several outbreaks were mentioned in which milk or milk products could have been the cause of food poisoning (Becker *et al.* 1994; Notermans *et al.* 1997). The poisoning, however, seems to be underestimated in the literature and statistics because of its short duration and because it might have been assumed, by mistake, as having been caused by *S. aureus* or *C. perfringens* enterotoxins (Granum *et al.* 1995). Symptoms such as nausea, diarrhea, and vomiting appeared within 7 h after consumption in 70% of the persons involved.

Relationships between the sources of contamination and the growth of *B. cereus* and its toxin production in pasteurised fluid milk produced in various countries were analysed by Eneroth *et al*. (1998), Christiansson *et al.* (1998), Larsen and Jørgensen (1997), Lin *et al.* (1998), Te Giffel *et al.* (1997), and Helmy *et al*. (1984). A predictive approach was used to describe the behavior of *B. cereus* in pasteurised milk (Baker & Griffiths 1993; Zwietering *et al.* 1996). Comprehensive risk assessment studies regarding *B. cereus* were published by Notermans and Batt (1998) and Notermans *et al.* (1997, 1998). Based on the data of the literature sources mentioned above, the real infectious dose may vary from about 1×10^5–1×10^8 viable cell or spores/g and hence food containing > 1×10^4 *B. cereus*/g may not be safe for the consumption.

Te Giffel *et al.* (1997) isolated *B. cereus* from 133 (40%) of the samples of pasteurised milk kept in household refrigerators in the Netherlands. They found low *B. cereus* counts, less than five per ml in 258 (77%), in the samples that were stored below 7°C. Other studies also revealed that organism could multiply rapidly in milk, especially under the storage conditions with temperatures above 7°C. For instance, Larsen and Jørgensen (1997) detected *B. cereus* in 120 of 257 samples of pasteurised milk. The viable count of *B. cereus* obtained was in the range between 10^3 and 3×10^5 cfu/ml.

This study was initiated to satisfy the need for data, which could be used as the background information in hazard analysis. Another purpose of the study was to examine the shelf life of pasteurised milk produced by the dairy plants in this country. For this reason, the growth of *B. cereus* naturally present in pasteurised milk as well as the microbial aspects of this product were monitored in view of the effect upon them of the storage temperature.

MATERIALS AND METHODS

Pasteurised milk and its sampling. Pasteurised milk was heated in plate heat exchangers at 74°C for 20 s and aseptically packaged in 1 l Tetra Brik cartons. Samples of commercially processed pasteurised milk (1.5% fat) were taken immediately after packaging and were kept at 5.0 ± 0.5°C during their transportation (shorter than 60 min) to the laboratory.

Examination procedure and testing. Whole packages of pasteurised milk were stored in incubators at 5, 7, 9, 11, and 13°C (± 0.5°C). *B. cereus* cell counts and total plate counts were obtained using 15 ml aliquots taken aseptically from each milk sample at each storage temperature at predetermined time intervals. Two or three milk samples were included in the experiment at each storage temperature. Experiments were duplicated.

Since low numbers of *B. cereus* were expected, 0.4 ml volumes of the milk samples were used as inoculums for each of > 20 Petri dishes containing Cereus Selective Agar growth medium (Fluka Chemie 22310, Buchs, Switzerland). The inoculated Petri dishes were incubated at 30°C for 24 h and pink colonies surrounded by a zone of precipitation were counted. According to STN ISO 7932: General guidance for the enumeration of *Bacillus* *cereus* (1998), the identities of all suspect colonies were confirmed by testing for glucose fermentation, Voges-Proskauer reaction, nitrate reduction, and β-hemolytic activity. *B. cereus* strains should be positive for all four reactions.

Total plate counts were determined at appropriate dilutions of pasteurised milk on plate count agar (PCA; Merck 5463, Darmstadt, Germany) incubated at 30°C for 72 h as specified in the ISO procedure (STN ISO 4833: General guidance for the enumeration of microorganims 1997).

Fitting of growth curves and calculating growth parameters. The growth function of Baranyi *et al.* (1993) was applied to fit the growth curves with the data observed. Thus, it was possible to model both the limited and the limitless growths, i.e. curves with or without an upper asymptote. The second stage of modelling involved the use of growth parameters calculated from the primary model in the modified Arrhenius-type model (Davey 1989) and the square root model (Ratkowsky *et al.* 1982). The first model was used to describe the effect of temperature on the lag-phase duration, and the second on the growth rate.

Growth parameters, lag-time, and specific growth rate calculated from the secondary models were used for the predictions of safety and shelf life of pasteurised milk stored at 5, 7, 9, 11, and 13°C. Both parameters (safety and shelf life) were defined as the time required for *B. cereus* counts and total plate counts to reach the density of 1×10^4 and 5×10^4 cfu/ml, respectively.

Prediction of the shelf-life and its validation. The time predictions for *B. cereus* numbers as well as for total plate counts to reach the level of 10^4 cfu/ml and 5×10^4 cfu/ml, respectively, are based on the growth curves found at each storage temperature. They were calculated from the Ratkowsky and modified Arrhenius equations describing the growth rate and lag-phase dependencies on temperature. The coefficients of the equations were found by regression analysis.

Reliability of both models used in this work was quantified by bias and accuracy factors introduced by Ross (1996) and evaluated by Baranyi *et al.* (1999). Accuracy factor for discrete case was estimated by:

$$A_f = \exp\left(\sqrt{\frac{\sum_{k=1}^{m}\left(Lnf\left(x^{(k)}\right) - Ln\mu^{(k)}\right)^2}{m}}\right)$$

and bias factor by:

$$B_f = \exp\left(\frac{\sum_{k=1}^{m}\left(Lnf\left(x^{(k)}\right) - Ln\mu^{(k)}\right)}{m}\right)$$

where: $Lnf(x^{(k)})$ – natural logarithm of the parameter from the model f

$Ln(\mu^{(k)})$ – natural logarithm of the parameter resulting from the observation (e.g. μ = specific growth rate or other)

m – the number of observation

RESULTS AND DISCUSSION

All pasteurised milk samples were plated on the day of production. The initial counts of *B. cereus*

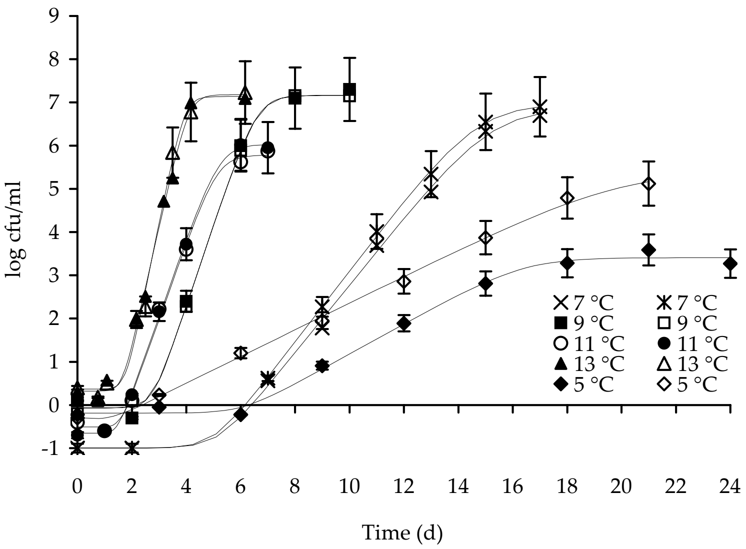

Figure 1. Growth of *Bacillus cereus* in pasteurised milk at 5, 7, 9, 11, and 13°C, respectively. Deviations of the storage temperatures were within ± 0.5°C

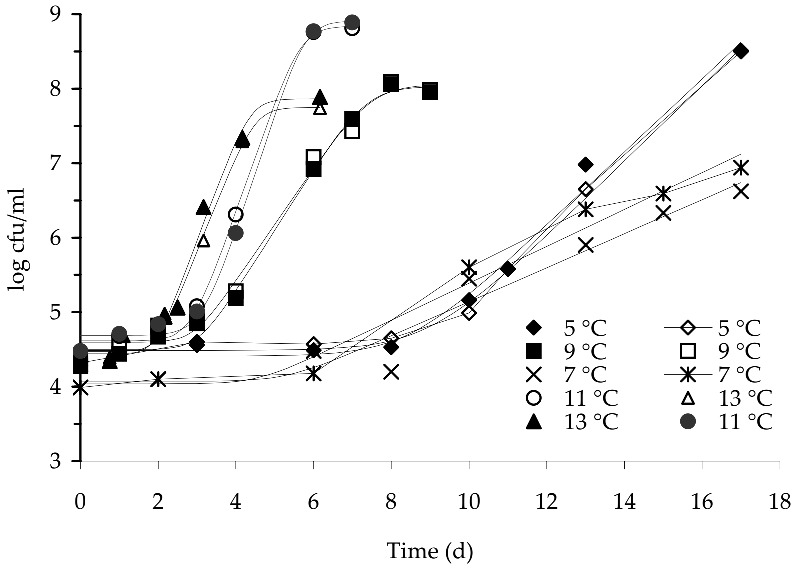

Figure 2. Growth of "total plate count" in pasteurised milk at 5, 7, 9, 11, and 13°C, respectively. Deviations of the storage temperatures were within ± 0.5°C

in the samples of pasteurised milk (n = 15) varied from their absence in 4 ml to 2.7 cfu/ml. The initial total plate counts in pasteurised milk were within the range of 1.1×10^4 to 3.0×10^4 cfu/ml (n = 15). Both these initial numbers of *B. cereus* and total bacteria were similar to the counts determined in pasteurised milk by Burden *et al*. (1995), Notermans *et al*. (1997) or Te Giffel (1997).

Growth dynamics of *B. cereus* and of the total bacterial flora in the pasteurised milk samples analysed during their storage are demonstrated in Figures 1 and 2, respectively. These figures present the time and temperature relations between *B. cereus* and the total plate counts that were used in the shelf life predictions for pasteurised milk. Obviously, relevant differences were found in the duration of the lag-phase for both *B. cereus* and the total plate counts determined at temperatures above 9°C as compared to temperatures below 7°C. The results thus confirmed that the temperature as low as 7°C delayed the onset of the bacterial growth of both the psychrotrophic bacteria and the toxigenic *B. cereus* (Figure 1). With high probability, this finding can be related to the differentiation between the non-psychrotrophic and psychrotrophic bacterial strains and their ability to grow below 7°C. In the case of temperature of 9°C, mesophilic *B. cereus* and mesophilic bacterial population present in milk

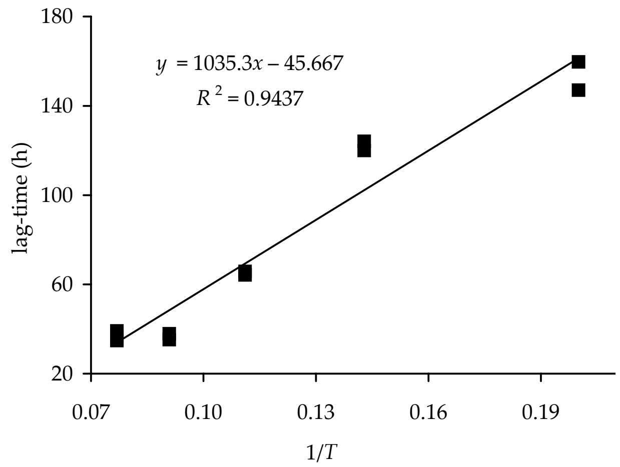

Figure 3. Lag-time of *B. cereus* strains presented in milk versus reciprocal storage temperature. Storage tests were carried out at 5, 7, 9, 11, and 13°C

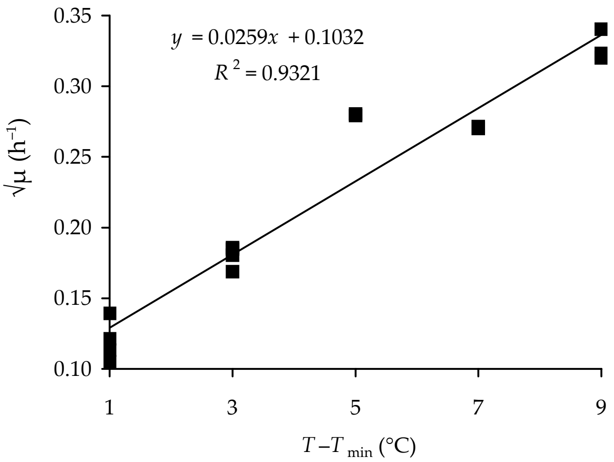

Figure 4. Square root growth rate of *B. cereus* strains presented in milk versus storage temperature (5, 7, 9, and 13°C; μ = growth rate)

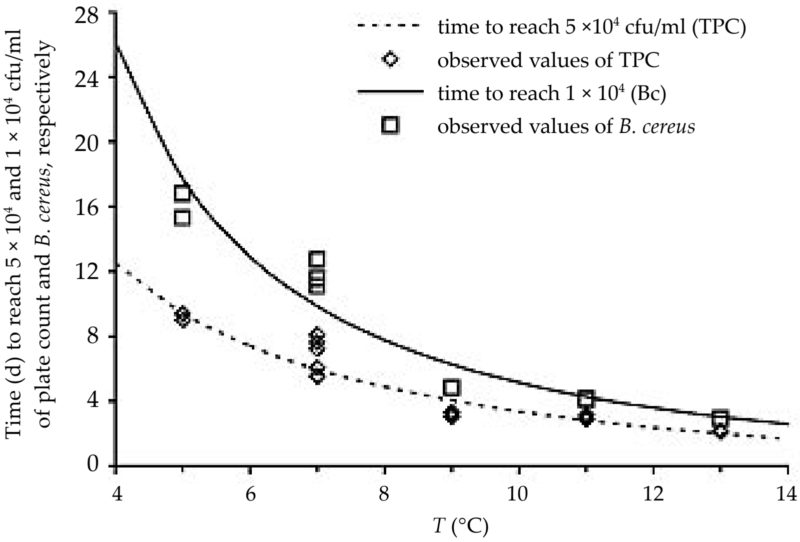

Figure 5. Predicted (lines) and observed (empty symbols) times to reach the levels of 1×10^4 and 5×10^4 cfu/ml for *B. cereus* and total plate count (TPC) in pasteurised milk in the dependence on the storage temperature, respectively

could also contribute to higher growth dynamics represented by the corresponding growth curves. This fact is important from the practical point of view and is obviously manifested in a shorter shelf life of pasteurised milk.

The results of the parallel trials (n = 2 or 3) at each temperature specified above were analysed mathematically. They enabled us to describe the effect of temperature on lag-phase of *B. cereus* (λ) by the modified Arrhenius equation ($\lambda = 1035.3/T - 45.667$; $R^2 = 0.9437$; Figure 3). The growth rate (μ) increased according to the Ratkowsky square root model ($\sqrt{\mu} = 0.0259\ (T - T_{min}) - 0.1032$; $R^2 = 0.9321$; Figure 4), (Ratkowsky *et al.* 1982).

Time predictions for *B. cereus* numbers as well as for total plate counts to reach the relevant safety level of 10^4 cfu/ml in the first case and the quality limit of 5×10^4 cfu/ml in the second case are presented in Figure 5. They are based on the growth curves plotted in Figures 1 and 2 and calculated from the above modelling equations. The predicted time to reach the quality limit of the total plate count was shorter than the time for *B. cereus* to reach 10^4 cfu/ml at each temperature. All these predictions were compared with the values observed. The results of their comparison confirmed that the shelf-life at 5 and 7°C was longer than declared by the producer. For example, *B. cereus* reached 10^4 cfu/ml approximately after a double period as compared to the shelf-life of the pasteurised milk as declared by the producer. In the case of proper cooling, the results indicate that other psychrotrophic bacteria can be more competitive than *B. cereus* and spoil the pasteurised milk before *B. cereus* has reached the counts higher than 10^4 cfu/ml. In this case, sensory changes of spoiled pasteurised milk should serve as a natural barrier to consumption. This fact, however, was not observed during the storage of pasteurised milk samples at 9, 11, and 13°C when the lag-phase of *B. cereus* was much shorter and the growth was more rapid. The predicted times for *B. cereus* to reach the limit 10^4 cfu/ml and the plate counts to reach the density of 5.10^4 cfu/ml were closer to each other at higher storage temperatures (Figure 5). The predictions presented were validated according to Baranyi *et al.* (1999) resulting in accuracy factors $A_f = 1.17$ and 1.25, and bias factors $B_f = 0.97$ and 1.04 for *B. cereus* and plate counts, respectively. The results confirmed that in the case of insufficient cooling represented by the temperatures of 9, 11, and 13°C in our experiments, *B. cereus* naturally present in pasteurised milk poses problems in view of not only quality but also safety.

The final count of *B. cereus* in pasteurised milk at the time of consumption is, of course, also dependent on the initial count of *B. cereus*. Notermans *et al.* (1998) pointed out that the initial count of *B. cereus* in pasteurised milk should be less than 1 cfu/ml. In such case the limit should not be exceeded within 7 d at 7°C. In our case, the initial count of *B. cereus* varied around the value recommended by Notermans *et al.* (1998). We should point out that our experiments were carried out at a time when the shorter 5 d shelf-life of pasteurised milk was permitted. At present, the shelf life of pasteurised milk in Slovakia was extended to 7 d.

Conclusion

The approval of shelf-life of pasteurised milk was based on a predictive approach in the temperature range of 5 to 13°C. The results confirmed that storage of pasteurised milk at 9°C and above led to unacceptable growth of *B. cereus* and concomitant bacterial flora. Safety and declared shelf-life of pasteurised milk were not achieved at this temperature within 5 days. The results found at the temperatures below 7°C gave evidence that psychrotrophic bacteria grew in the milk to counts causing the spoilage of milk before the toxigenic *B. cereus* could have reached the limit of 10^4 cfu/ml and produced enterotoxins.

In spite of the fact that the producer is responsible for the safety of pasteurised milk, it is evident that this responsibility has to be shared with the retailer and consumer by applying proper cooling, especially in the summer period.

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Súhrn

Valík Ľ., Görner F., Lauková D. (2003): **Dynamika rastu** *Bacillus cereus* **a trvanlivosť pasterizovaného mlieka**. Czech J. Food Sci., **21**: 195–202.

Sledovali sme dynamiku obsahu *Bacillus cereus* v pasterizovanom mlieku v závislosti od teplôt ich uchovávania (5, 7, 9, 11 °C). Obsah *B. cereus* bol stanovený v intervale od jeho neprítomnosti v 4 ml po 2,3 KTJ/ml. Počiatočný celkový počet mikroorganizmov bol v pasterizovanom mlieku v rozmedzí $1,1 \times 10^4$ až $3,0 \times 10^4$ KTJ/ml ($n = 15$). Na základe rastových kriviek a z nich vyplývajúcich parametrov rastu *B. cereus* sa zistilo, že tento druh v pasterizovanom mlieku rástol aj pri teplotách 5 a 7 °C. Pre analýzu rastu *B. cereus* v závislosti od teploty uchovávania bol použitý odmocninový model ($\sqrt{\mu} = 0,026\ (T - T_{min})$; $R^2 = 0,93$). Lag-fáza bola opísaná modifikovanou rovnicou Arrheniovho typu ($\lambda = -45,667 + 1035,3/T$; $R^2 = 0,94$). Porovnania predikcie času pre dosiahnutie počtu *B. cereus* 10^4 KTJ/ml v pasterizovanom mlieku a celkového počtu mikroorganizmov $5,0 \times 10^4$ KTJ/ml poukázali na skutočnosť, že limitný celkový počet mikroorganizmov bol dosiahnutý vo všetkých pokusoch skôr ako limitný počet *B. cereus*. Táto skutočnosť vedie k poznatku, že pasterizované mlieko sa s najväčšou pravdepodobnosťou znehodnocuje rastom saprofytických baktérií predtým, ako *B. cereus* dokáže vytvoriť nebezpečné koncentrácie enteretoxínov.

Kľúčové slová: pasterizované mlieko; *B. cereus;* trvanlivosť

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