Food Microbiology, 1997, **14**, 143–151

ORIGINAL ARTICLE

A risk assessment study of *Bacillus cereus* present in pasteurized milk

S. Notermans[1]*, J. Dufrenne[1], P. Teunis[1], R. Beumer[2], M. te Giffel[2] and P. Peeters Weem[3]

Due to the heat resistance of Bacillus cereus*, its potential pathogenic character, the capability to grow in milk and reported diseases upon consumption of dairy products, the organism should be considered as hazardous in pasteurized milk. Human exposure experiments based on (1) enquiring about the storage conditions (time and temperature) of pasteurized milk in households in The Netherlands and (2) performing of storage tests at 6, 8, 10 and 12°C, respectively, demonstrated the following: pasteurized milk is consumed within 2–12 days after pasteurization and is stored at temperatures varying from <5–13°C. Visually spoiled milk is not consumed anymore. Based on the distribution of both the storage time and temperature of milk in private households, approximately 7 and 4% of the total portions of milk consumed in The Netherlands could contain $>10^5$ and $>10^6$* B. cereus *ml^{-1}, respectively. Taking into account the high portion of milk consumed (approximately 10^9–10^{10} portions/year) and the absence of epidemiological evidence that milk is causing foodborne diseases due to* B. cereus*, the dose–response relationship of this organism may need to be considered. Results of the storage tests, based on the worst case situation, provided useful information on the effect of the risk factors, storage temperature and time, to human exposure. However, such information can be obtained more easily by applying predictive models. Models also enable us to find out the initial number of spores which can be considered as a third risk factor.*

Received: 15 July 1996

[1]*National Institute of Public Health and the Environment, P.O. Box 1, 3720 BA Bilthoven, The Netherlands*
[2]*Agricultural University of Wageningen, P.O. Box 8129, 6700 EV Wageningen, The Netherlands*
[3]*Regional Inspectorate for Health Protection, Oostergoweg 2, 8932 PG Leeuwarden, The Netherlands*

Introduction

Over the last 20 years, there has been increased activity in the development of concepts for production of safe food to protect the consumer. Now, it becomes increasingly clear that the application of good manufacturing practices (GMP), followed by process control, based on the hazard analysis critical control point (HACCP) concept are prevailing. However, application of GMP/HACCP makes it explicit that absolutely safe food can not be produced, and that safety has economical consequences. Therefore, there is also a growing interest in the application of risk assessment, or at least elements of it, in the context of GMP/HACCP.

Risk assessment is a quantitative estimation of the probability of occurrence of a (foodborne) hazard. Information about the probability of occurrence of a hazard is becoming increasingly important for managerial decisions. However, it should be noted that at this moment little experience exists in the use of risk analysis in the production of safe food. So far, there have been only a few

*Corresponding author.

0740-0020/97/020143+09 $25.00/0/fd960076

formal, food-related, microbiological risk-assessment studies. One of these studies dealt with risk assessment of the use of cracked eggs (Todd 1996) and another study dealt with a health-risk assessment of *Listeria monocytogenes* (Farber et al. 1996). These studies demonstrate that risk-assessment results can easily be made subservient to HACCP. This especially applies to the so-called critical control points (CCPs) which have to be identified in the HACCP approach. At these CCPs, critical limits should be specified in order to meet declared end-product specifications (Notermans and Mead 1996). Or in other words, CCPs critical limits have to be set which guarantee an acceptable low risk that the final product may cause harm. For a (processed) perishable product, such as pasteurized milk, critical limits may be, for example, the storage time and temperature. Risk assessment can provide this information.

Regulatory agencies are involved in the demonstration that the producers are adhering to existing regulations and directives. They are also responsible for setting end-product specifications, which are the basis to which producers set their critical limits at CCPs. Now, with the introduction of the GMP/HACCP concept in food processing, inspection of the control system and guidance may become an additional task for the authorities. Guidance comprises, among others, taking the initiative in the process of communication between producers and consumers. A clear example is the consumer behaviour and the possible consequences for the safety of a food product in terms of setting critical limits at CCPs. Regulatory agencies may also benefit from risk assessment.

In this paper, a risk-assessment study of *Bacillus cereus* present in pasteurized milk is presented.

Materials and Methods

Definitions for risk assessment

Definitions used are based on the definitions recently presented by Notermans et al. (1996).

Risk assessment is the estimation of the probability that an adverse effect will occur to the consumer (in this special case caused by *B. cereus*, present in pasteurized milk) and is a component of risk analysis. The process of risk assessment consists of the following steps: (1) hazard identification, (2) exposure assessment, (3) dose–response assessment and (4) risk characterization.

Hazard identification is the identification as to whether *B. cereus* may have the potential to cause an adverse health effect upon consumption of pasteurized milk. For identification of *B. cereus* as a hazardous organism in pasteurized milk, the flow sheet as proposed by Notermans and Mead (1994) has been applied.

Exposure assessment is the quantitative estimation of the dose of *B. cereus* to which the consumers are exposed at time of consumption. To gain information on human exposure, storage tests and studies on the storage conditions of pasteurized milk in the home have been applied.

Dose–response assessment is the translation of exposure time into disease. It is the process of obtaining quantitative information on the probability of human illness following exposure to *B. cereus*. Dose–response relationships may be gained from epidemiological analyses of foodborne diseases caused by *B. cereus*.

Risk characterization is the process of identification and quantification of the factors involved in the risk of being exposed to unacceptable levels of *B. cereus* at time of consumption of the milk.

Enquiries carried out in households

Enquiries were carried out to obtain information regarding the storage conditions (time and temperature) of pasteurized milk in households. Two hundred and seventy-three private households were visited, and the storage times of opened jars of milk were determined. In addition, participants were asked whether visually-spoiled milk was consumed or not. The average temperature of refrigerators was measured in 1993 by the Regional Inspectorate for Health Protection in Leeuwarden (de Lezenne Coulander 1994).

They tested 125 refrigerators and their results have been used in this study.

Storage tests

Every 2 weeks, from March 1995 until February 1996, two packages of freshly-pasteurized milk (fat content 3%) were obtained from six different milk-processing plants in The Netherlands. The milk was produced on Monday and delivered at the laboratory on Tuesday having been stored at 6°C. Immediately after delivery (day 1), two packages of one processing plant were chosen and mixed. The numbers of *B. cereus* present were estimated by using a most probable number (MPN) procedure. The rest of the milk was divided into four portions of 200 ml, put into 300 ml sterile Erlenmeyer flasks and placed in thermostatically-controlled water-baths at temperatures of 6, 8, 10 and 12°C, respectively. At different intervals, depending on the storage temperature, the numbers of *B. cereus* present were counted using a plating technique, until visual spoilage (coagulation) was observed. The maximal storage time was 24 days. In total, 38 mixed samples, six to seven samples from each plant, were used in the storage tests.

Enumeration of B. cereus *present in milk*

MPN. From every milk sample at day 1, portions of 3×100 ml, 3×10 ml and 3×1 ml were taken and incubated at 30°C. After incubation for 18±2 h, the portions were plated on mannitol–egg yolk–polymyxin agar (MEYP, Mossel et al. 1967) and incubated at 30°C for 18± 2 h. From each plate with typical colonies resembling *B. cereus*, three colonies were picked and streaked on to tryptone soya agar (TSA) plates and then incubated at 30°C for 18±2 h. From the TSA plates, brain heart–infusion (BHI) broth tubes were inoculated, and incubated at 30°C for 24±2 h. Four confirmation tests were performed on the *B. cereus* in the BHI tubes: Voges–Proskauer (+); gelatine hydrolysis (+); glucose fermentation (+) and nitrate reduction (+). The numbers of *B. cereus* were then estimated by using the MPN method of De Man (1974).

Numbers. From day 2, the milk was tested for the number of *B. cereus* by preparing 10-fold dilution of the milk in peptone physiological saline (PPS) and plating out these dilutions on MEYP followed by incubation at 30°C for 18±2 h. Typical colonies were tested as for the MPN, by testing three colonies for each sample dilution that presented countable colonies.

Enumeration of the total count present in milk

To determine the total bacterial count present in stored milk samples, 10-fold dilutions of milk in PPS were plated on plate count agar (Difco). The plates were incubated at 30°C for a period of 3 days.

Results

Hazard identification

B. cereus is a well-established, foodborne pathogen. Analysis of reported foodborne diseases reveals that the organism is frequently diagnosed as the cause of gastrointestinal disorders (Kramer and Gilbert 1989). The organism is ubiquitous in nature, and as a consequence, present in animal feed and fodder (Kramer and Gilbert 1989). Due to its rapid sporulating capacity, the organism easily survives in the environment and during intestinal passage in cows. The organism contaminates raw milk via faeces and soil. It has been demonstrated that additional sources of milk contamination are the pipelines and milk tanks at the processing plants (Van Heddeghem and Vlaemynck 1993; Te Giffel et al. 1996).

Due to their heat-stable spores, *B. cereus* easily survives the pasteurization process. Results obtained in this study revealed that day-old pasteurized milk contained about 10 *B. cereus* organisms per 100 ml (Table 1) and that all 38 samples of each two 1 l packages contained *B. cereus*. The number of spores present seems to be seasonally dependent with the highest contamination occurring during the grazing period (Meer et al. 1991, Te Giffel et al. 1995). From literature analy-

sis of consumer illnesses, it is evident that dairy products account for a substantial proportion of cases of foodborne diseases. Several outbreaks have been reported in which milk or milk-related products containing *B. cereus* were suggested to be the cause of the disease (Bannerjee and Black 1986, Bryan 1983, Bulyba et al. 1973, WHO Surveillance Programme Report 1995). Analysis of *B. cereus* strains isolated from milk revealed that almost all strains tested produced enterotoxin (Granum et al. 1993, Ogihara et al. 1992, van Netten et al. 1990). It has also been claimed that the toxin is produced at all storage temperatures of milk allowing growth of *B. cereus* (Sutherland 1993).

To cause disease, *B. cereus* has to proliferate in pasteurized milk. *B. cereus* is an autotrophic organism, and does not require a rich substrate for multiplication. Several reports have shown that the organism can proliferate rapidly in milk, depending on the storage temperature, (Christiansson et al. 1989, Helmy et al. 1984, Crielly et a. 1994) and some of these reports indicate the production of enterotoxin in milk by *B. cereus*. Recent studies revealed that the organism will grow at temperatures as low as 5°C (Meer et al. 1991). As a consequence of all the above mentioned aspects, the presence of *B. cereus* in milk should be considered as hazardous.

Exposure assessment

To assess human exposure to *B. cereus* present in pasteurized milk, storage tests were carried out. Additionally enquiries were made to learn the storage conditions of pasteurized milk in domestic households.

Enquiries made in households. The enquiries revealed that the storage time of pasteurized milk varied from 2–12 days. The frequency distribution of the storage time is

Table 1. Initial numbers of *B. cereus* present in milk 1 day after pasteurization as determined using a most probable number method applying three 10-fold dilutions in triplicate

Period of sampling	Number of samples	*B. cereus* count ($\log_{10}$ cfu 100 ml^{-1})
March–June, 1995	12	0·46±0·046[a]
July–October, 1995	16	1·40±0·47
November–February, 1996	12	0·93±0·29
Total	38	0·96±0·57

[a]Standard deviation.

Table 2. Storage time of pasteurized milk in The Netherlands

Storage time (days after pasteurization of the milk)	Number of samples	P_t (Probability that milk is stored for the indicated time)
1	0	0
2	6	0·022
3	16	0·059
4	43	0·158
5	43	0·158
6	33	0·121
7	39	0·143
8	42	0·154
9	15	0·055
10	12	0·044
11	14	0·051
12	10	0·037
13	0	0
Total	273	1·000

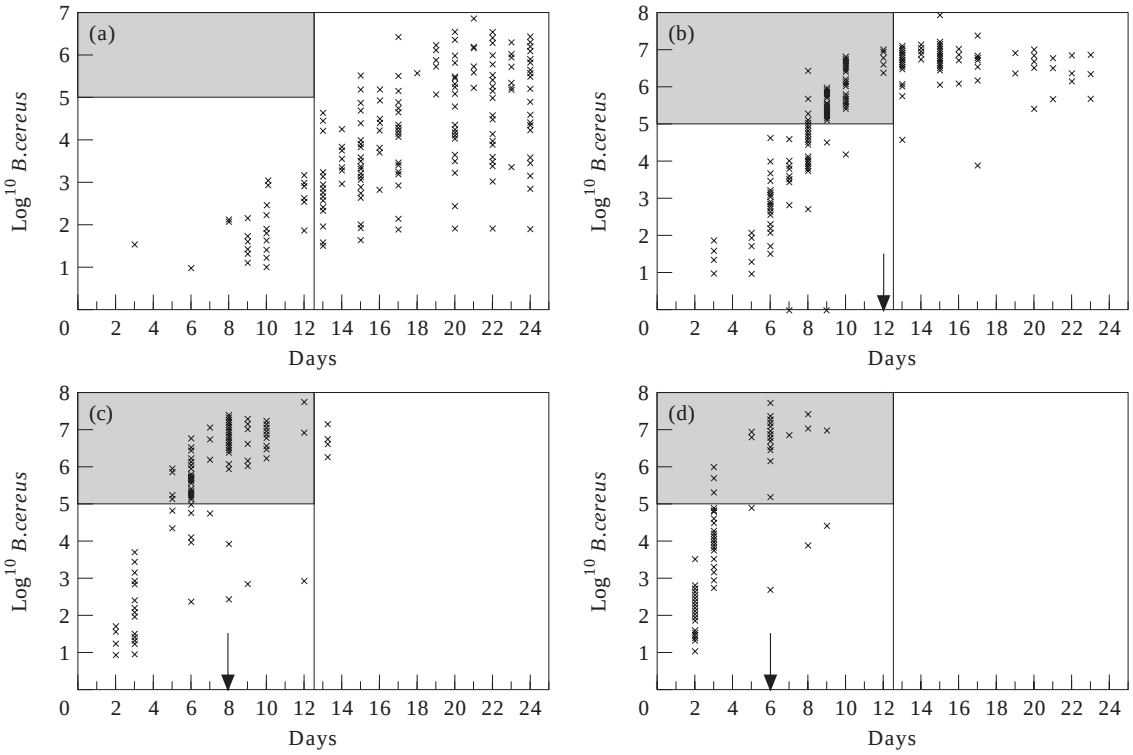

Figure 1. Results of storage tests carried out with pasteurized milk. Portions of pasteurized milk were stored at 6 (Fig. 1(a)) 8 (Fig. 1(b)), 10 (Fig. 1(c)) and 12°C (Fig. 1(d)), respectively. From day 2, the incubated milk samples were tested for the numbers of *B. cereus* ml^{-1} until obvious spoilage occurred. The arrows indicate the first samples showing spoilage. Spoiled samples were not tested further and have been excluded. The shaded areas in the figures indicate the consumption period and the samples containing $>10^5$ *B. cereus* ml^{-1}.

presented in Table 2. It can be seen that the frequency distribution does not show a normal distribution. A clear 'shoulder' is seen at the end of the storage period.

Table 3 shows the frequency distribution of the temperatures of refrigerators in Dutch private households.

Storage tests. Results of the storage tests are presented in Fig. 1(a)–(d). At each of the incubation temperatures the number of *B. cereus* increased during the storage period. Maximum numbers, exceeding 10^7 cfu ml^{-1}, were observed before overt spoilage was observed. The shaded areas in the figures

Table 3. Average storage temperatures of pasteurized milk in The Netherlands (de Lezenne Coulander, 1994)

Temperature (°C) of refrigerators used to store milk	Numbers of refrigerators within the indicated temperature range	P_T (probability that the milk is stored at the indicated temperature range)
<5	37	0·296
5–<7	52	0·416
7–<9	32	0·256
9–<11	2	0·016
11–<13	2	0·016
Total	125	1·000

indicated the period of consumption where numbers of *B. cereus* exceeded 10^5 cfu ml^{-1}.

Spoilage of milk was not observed at a storage temperature of 6°C. At 8, 10 and 12°C the first samples showing spoilage were observed after 12, 8 and 6 days, respectively. Because spoiled milk was not consumed, these samples were excluded from the results.

It was observed that at incubation temperatures of 6, 8 and 10°C, the total bacterial count was similar to the *B. cereus* count (results not presented), suggesting that *B. cereus* was the dominant bacterial flora present. At the incubation temperature of 12°C, the total count was much higher than the number of *B. cereus* present (results not presented).

Exposure calculations. From the results presented in Tables 2 and 3 and in Fig. 1(a)–(d), the probability was calculated that milk at time of consumption contained $>10^5$ cfu ml^{-1}. For this calculation, the results of the storage tests between 2 and 13 days of storage (consumption period) were the starting point. From this part, the fraction was taken containing samples with a count of $>10^5$ ml^{-1} *B. cereus* (in Fig. 1(b) it is the fraction between 7 and 13 days of storage and amounts of 0·634). These fractions were multiplied with the probability that milk is stored between 5–7, 7–9, 9–11 and 11–13°C, respectively (for storage between 7–9°C this fraction is 0·256, Table 3) and multiplied with the probability that the milk is stored between the indicated days (for a storage period between 7 and 13 days this fraction is 0·341, Table 2). The results of these calculations are presented in Table 4. It reveals that at time of consumption approximately 7% of the portions of pasteurized milk consumed contained $>10^5$ cfu *B. cereus* ml^{-1}. The same calculations were made for other levels of contamination and are presented in Table 5.

In The Netherlands there are about 15×10^6 consumers of pasteurized milk. If each of them drink 50–500 portions of 100 ml of milk per year, about 10^9–10^{10} portions of 100 ml of milk are consumed. The calculations suggest that 7 and 4% of these portions contain $>10^5$ and $>10^6$ *B. cereus* ml^{-1}, respectively.

Dose–response assessment

Kramer and Gilbert (1989) summarized a large number of foodborne outbreaks caused by *B. cereus*. The results showed that the levels of *B. cereus* present in foods causing a 'diarrhoeal syndrome' varied from $1{\cdot}2\times10^3$–10^8 organisms g^{-1}; the median values was around 10^7 organisms. Levels of *B. cereus* present in food causing an 'emetic syndrome' varied from $<10^4$–$>10^{10}$ g^{-1} with a medium value of $1{\cdot}0\times10^7$ g^{-1}.

Infection experiments have also been carried out with animals and human volunteers. Cats and dogs fed experimental diets containing more than 10^5 *B. cereus* g^{-1} developed severe diarrhoea and dehydration (Nikodemusz 1967). The human volunteer study carried out by Hague (1995) supported the notion that *B. cereus* also induced diarrhoea in humans. However, Dack et al. (1954), failed to produce food-poisoning symp-

Table 4. Calculation of the probability that pasteurized milk contains $>10^5$ *B. cereus* ml^{-1} at time of consumption

Storage temp. (°C) (and range)	6 (5–<7)	8 (7–<9)	10 (9–<11)	12 (11–<12)
Fraction[a] $>10^5$ ml^{-1}	0	0·634	0·755	0·431
P_T[b]	0·416	0·256	0·016	0·016
P_t[c]	0	0·341	0·762	0·847
$P>10^5=\Sigma$ (Fraction$\times P_T\times P_t$)	0	0·055	0·009	0·006=0·07

[a]Fraction of the milk containing $>10^5$ *B. cereus* ml^{-1} in the period of consumption (see Fig. 1(a)–1(d)).
[b]Fraction of the milk stored in the related temperature range (see Table 3).
[c]Fraction of the milk stored in the related period (see Table 2).

toms with *B. cereus*. Recently, Langeveld et al. (1996) carried out a quantitative human volunteer study. During a period of 3 weeks the participants were exposed to *B. cereus* by drinking portions of pasteurized milk which had been stored for 3–14 days at 7·5°C. The total numbers of *B. cereus* ingested varied in that study from 10^5–>10^8 organisms (10^3–> 10^6 ml^{-1}). No indications were obtained that these organisms caused harm to the volunteers. The discrepancy between reported and analysed outbreaks, and certain human volunteer studies may be explained by assuming that not all strains of *B. cereus* have the potency to cause food-poisoning.

In regulations of most European countries, numbers of 10^4–10^5 cfu *B. cereus* ml^{-1} of a product at expiry date, are set as critical limits for acceptance of a particular food. Also for diagnosing foodborne disorders resembling those caused by *B. cereus*, numbers of *B. cereus* >10^5 are regarded as highly indicative.

From a hygienic point of view, the presence of *B. cereus* in food in excess of 10^5/10^6 organisms g^{-1} is considered not consistent with the generally-accepted principles of good food practice.

In conclusion, *B. cereus* is a well-recognized pathogenic organism. Although, no clear dose–response data are available, and there are suggestions that *B. cereus* may not always cause harm (Dack 1954, Langeveld et al. 1996), it has been considered that a number of 10^5 (toxigenic) *B. cereus* present in pasteurized milk should be regarded as hazardous to the consumer. Our results demonstrate that 7% of the portions of milk at time of consumption could show a *B. cereus* count exceeding this value.

Table 5. Exposure to *B. cereus* after consumption of pasteurized milk (based on the calculations as demonstrated in Table 4)

Exposure dose (cfu ml^{-1})	Probability
>10	99%
>10^2	21%
>10^3	14%
>10^4	11%
>10^5	7%
>10^6	4%
>10^7	<1%

Risk characterization

The main factors involved in the risk of being exposed to 'unacceptable' levels of *B. cereus* at time of consumption of milk comprise the initial count of spores present, the storage temperature and the storage time. In this study, only information about the effect of storage time and storage temperature were assessed. Table 6 presents the effect of both factors on a final level of 10^5 cfu *B. cereus* ml^{-1} of milk. The calculations are based on the worst-case conditions. In The Netherlands, pasteurized milk has to be stored at ≤7°C for maximal 7 days. Under these conditions the number of *B. cereus* present at expiry date will not be >10^5 cfu ml^{-1}. Table 6 also shows predicted results based on the mathematical model applied by Zwietering et al. (1996). Comparing the results reveals that both approaches provide fairly equal results. It has to be noted that in the model of Zwietering et al. (1996) the lag phase was not taken into account.

Discussion

In this paper the formal process of risk assessment has been worked out in detail for *B. cereus* and pasteurized milk. This example was chosen because most aspects of risk assessment are represented.

Table 6. Time in days for *B. cereus* to multiply to 10^5 cfu ml^{-1} at different temperatures using storage tests and predictive microbiology, based on the most worse case situation

Method	6°C	8°C	10°C	12°C
Storage tests[a]	13·8	6·8	4·2	2·6
Predictive microbiology[b]	12·9	7·2	4·6	3·2

[a]Initial count of *B. cereus* 0·09 ml^{-1} (see Table 1).[b]Based on the model of Zwietering et al. (1996); initial count of *B. cereus* 0·1 ml^{-1}.

The hazard-identification study clearly revealed that *B. cereus* present in milk may cause harm to the consumer. As a consequence, assessment of human exposure to *B. cereus* via consumption of milk was justified.

Exposure analysis was carried out based on household enquiries regarding storage conditions of pasteurized milk and on storage tests of milk at several incubation temperatures. In the calculations, storage times and storage temperatures were considered to be independent, which is obviously not true. It may be assumed that milk stored at a high temperature will be stored for a shorter period simply due to spoilage. However, visually-spoiled milk is not consumed and was excluded from the calculations.

For interpretation of the human exposure, it is necessary to have quantitative information about the consequences of being exposed. In this study, the necessary information was obtained from a literature survey and collection of additional information. Results obtained revealed that although *B. cereus* can cause foodborne illness, no clear results are available to estimate a dose–response relationship. Additionally, there exist evidence that not all *B. cereus* will cause foodborne illness (Dack et al. 1954; Langeveld et al. 1996). Based on analysis of foodborne illness caused by *B. cereus* in the past, safety criteria have been set; these vary from 10^4–10^5 g^{-1} of a food product. Our calculations demonstrate that 11% of the portions of milk consumed in The Netherlands contains $>10^4$ *B. cereus* ml^{-1}, while 7% of the portions contain $>10^5$ ml^{-1}. The results obtained clarified that a substantial portion of consumed pasteurized milk does not meet the legal criteria at time of consumption. It has been demonstrated that the storage conditions are an important factor, and that Dutch consumers do not always meet the prescribed storage conditions.

To carry out a proper risk-assessment study more information is needed to evaluate human exposure to *B. cereus*. Among others information need to be collected about the toxigenity of strains. In the absence of real quantitative information, it is not possible to estimate the consequences of being exposed to *B. cereus* via milk consumption. Therefore, for the present it remains advisable to adhere to existing regulations on maximal numbers of *B. cereus* present in ready-to-eat food.

Risk characterization provides information about the factors involved in unacceptable exposure to *B. cereus* via consumption of pasteurized milk. Factors that clearly determine the risk of being exposed to an excessive number of *B. cereus* are the initial count after pasteurization, the storage time and the storage temperature. The present study only provides information about the effect of storage time and the storage temperature. However, such information can also be obtained by applying predictive methodology, as has been demonstrated recently by Zwietering et al. (1996). Comparing the results obtained applying the model used by Zwietering et al. (1996), and the results found in this study based on storage testing reveals that definitive information is as yet not attainable. The main reason is that the model of Zwietering et al. (1996) is based on the most worse-case situation. However, the model allows at least calculations of the relative contributions of several risk factors, including the effect of the initial count of *B. cereus*.

Considering that consumption of pasteurized milk containing $>10^5$ cfu of *B. cereus* ml^{-1} may cause harm to the consumer suggests that the expiry date of pasteurized milk produced in The Netherlands can be set at 7 days based on a storage temperature of 7°C. Although these conditions have been made mandatory in the past a discussion between producers of pasteurized milk and consumer organizations should be initiated how to improve the possible undesired situation of being exposed to an excessive number of *B. cereus*, as is demonstrated in this research.

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