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Biodiversity in *Bacillus cereus*

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Het rapport in het kort

In het kader van een EU project zijn door de verschillende partners experimenten uitgevoerd om inzicht te krijgen in de variatie in eigenschappen van *B. cereus* stammen welke bijdragen aan de mate van virulentie. Hiertoe zijn 100 *B. cereus* stammen geselecteerd en eigenschappen zoals toxine vorming, hitteresistentie, ontkieming en groeicurves bepaald. De experimentele gegevens zijn in dit rapport samengevat en geanalyseerd om de biodiversiteit binnen en tussen bepaalde karakteristieke *B. cereus* groepen in kaart te brengen. De 100 stammenset is daarvoor opgedeeld in 4 groepen, nl. omgevings-, voedsel-, diarree-, en braakstammen. Resultaten laten zien dat de biodiversiteit in *B. cereus* groot is, zowel tussen als binnen groepen. Doch, braakstammen vormen veelal een uitzonderlijke groep. Zij groeien niet bij temperaturen ≤ 7 °C en hun sporen zijn beter bestand tegen verhitting vergeleken met de andere groepen. Zij vormen dan ook snel een risico wanneer verhitte producten buiten de koelkast worden bewaard. Diarreestammen groeien juist wel bij 7 °C en, hoewel hun sporen minder resistent zijn, ze overleven nog steeds pasteurisatieprocessen. Zij kunnen groeien als koelkasttemperaturen de 7 °C bereiken, vooral in voedingsmiddelen met een neutrale pH. Wanneer normale voorzorgsmaatregelen in acht worden genomen zal de groei van deze stammen beperkt zijn, maar er is een smalle veiligheidsmarge. Statistische analyses wezen uit dat 1- goed gereguleerde koelkasttemperaturen (< 7 °C, ter voorkoming van groei diarreestammen tijdens bewaren), 2- het produceren van *niet* pH neutrale producten en 3- snel afkoelen (middels bijvoorbeeld. opdelen in kleine porties) van grote hoeveelheden voorbereid voedsel (ter voorkoming van groei braakstammen en daarmee gepaard gaande braaktoxine productie) bijdragen aan het voorkomen van voedselinfecties door *B. cereus* in Europa.

Trefwoorden: voedselmicrobiologie, voedselcriteria, contaminatieroute, braaktoxine, enterotoxine

Abstract

Experiments have been performed by different partners to identify variability in properties of *Bacillus cereus* strains that contribute to the extent of their virulence as part of an EU project. To this end, 100 *B. cereus* strains were selected and screened for biological properties, such as toxin production, heat resistance, germination and growth curves. This report gives a summary of the experimental data and subsequent analysis to identify the biodiversity within and between certain characteristic *B. cereus* groups. The 100 strain set was, therefore, divided into 4 categories, *i.e.* environmental, food, diarrhoeal and emetic strains. Results show that the biodiversity in *B. cereus* is large, both between and within groups. Yet, emetic strains often form a distinct group. They do not grow at temperatures ≤ 7 °C and their spores are more heat resistant compared to the other groups. Emetic strains will, therefore, form a risk when heated products would be kept outside the fridge. Diarrhoeal strains do grow at 7 °C and, although their spores are more heat resistant, still they will survive pasteurization processes. They can grow when fridge temperatures reach 7 °C, especially in food products having a neutral pH. So, growth of diarrheal strains can be prevented when standard safety measures are being considered, still, this safety margin is small. Statistical analyses revealed that 1) well regulated fridge temperatures (< 7 °C, to prevent growth of diarrhoeal strains during storage), 2) producing food products with a non-neutral pH value and 3) portioned chilling to rapidly lower the temperatures of precooked food (to prevent growth of emetic strains and, with that, emetic toxin production) will contribute to prevent *B. cereus* foodborne poisoning in Europe.

Keywords: food microbiology, hazardous strains, contamination route, emetic toxin, enterotoxin

Contents

Samenvatting

Algemeen: Dit rapport is tot stand gekomen in het kader van een EU project met als doel voedselvergiftiging door de bacterie *Bacillus cereus* in de toekomst te kunnen reduceren. *B. cereus* is een voedselgerelateerde pathogeen waarvan de eigenschappen die bijdragen aan de virulentie nauwelijks gekwantificeerd zijn. Dit is mede te wijten aan de grote biodiversiteit tussen *B. cereus* stammen. De meeste stammen zijn waarschijnlijk niet of mild pathogeen. Daarnaast bestaan echter ook zeer virulente stammen die hoge concentraties braak- en/of enterotoxines kunnen produceren.
Dit rapport: Doel van dit onderzoek is inzicht te krijgen in de biodiversiteit van *B. cereus* en met deze kennis te komen tot aanbevelingen ten aanzien van het reduceren van dit microbiologische voedselprobleem.
Methode: Biofysische eigenschappen welke experimenteel zijn onderzocht door partners binnen dit project omvatten onder andere minimum en maximum groeitemperatuur, toxineproductie, hitteresistentie, ontkieming en het bepalen van groeicurven voor een geselecteerde groep van 100 *B. cereus* stammen. Om potentiëel gevaarlijke stammen te kunnen identificeren zijn sommige data gebruikt om biologisch relevante parameterwaarden te schatten. Zo zijn decimale reductietijden (D-waarden) en tijd tot eerste decimale reductie (d-waarden) geschat uit hitteresistentiedata en zijn aangeleverde groeicurves gebruikt om lag-tijden en groeisnelheden te schatten. Om vervolgens te komen tot een waardevolle analyse met betrekking tot het identificeren van potentiëel belangrijke contaminatieroutes is de 100-stammenset opgedeeld in 4 groepen, te weten: diarree-, braak, omgevings- en voedselstammen.
Resultaten: Resultaten geven aan dat de biodiversiteit in *B. cereus* groot is zowel binnen als tussen groepen. Doch, braak- en diarreestammen vormen vaak een aparte groep met betrekking tot biologische eigenschappen in relatie tot voedselveiligheid. Braakstammen groeien namelijk niet bij temperaturen ≤ 7 °C. Daarnaast zijn hun sporen veel resistenter tegen hittebehandeling vergeleken met de andere groepen. Het buiten de koelkast bewaren van verhitte producten brengt dan ook een verhoogd risico op voedselvergiftiging door braakstammen met zich mee. Zo'n 75 % van de onderzochte diarreestammen groeien bij 7 °C en, ook al zijn hun sporen minder resistent, pasteurisatieprocessen kunnen ze overleven. Tevens is de groeisnelheid bij verschillende temperaturen optimaal wanneer het gaat om voedingsmiddelen met een neutrale pH.
Aanbevelingen: Statistische analyses hebben geleid tot de volgende aanbevelingen ten aanzien van het voorkomen van *B. cereus* voedselvergiftiging in Europa. Tijdens bewaren kan groei van diarreestammen voorkomen worden door koelkasttemperaturen beneden de 7 °C te houden. Op basis van minimale groeitemperaturen van braakstammen (≥ 10 °C) zal voorgekookt voedsel zo snel mogelijk afgekoeld moeten worden door middel van bijvoorbeeld geportioneerd koelen om groei, en daarmee gepaard gaande braaktoxineproductie, te voorkomen. Om directe bacteriegroei te voorkomen moet voedsel een *niet* neutrale pH hebben.

Summary

General: This report describes research within the framework of a EU project with a focus on the future prevention of food poisoning caused by the bacterium *Bacillus cereus*. Little is known about the characteristics that contribute to virulence of this food borne pathogen. This is partly due to the huge biodiversity in *B. cereus* strains. Most strains are presumably innocuous or mildly pathogenic. Still, some strains are able to produce high concentrations of emetic or enterotoxins.
This report: Main goal of this study is to get insight in the biodiversity of *B. cereus* and, with this knowledge, make recommendations on how to reduce this food microbiology problem.
Method: Partners in this project have performed several experiments in order to assess biophysical properties. For example, minimum and maximum growth temperatures, toxin production, heat resistance, germination and growth curves were assessed for a selected set of 100 *B. cereus* strains. Some of the data were used for parameter estimation to quantify biologically relevant properties. That is, decimal reduction times (D-values) and times until first decimal reduction (d-values) were estimated from heat resistance data. In addition, available growth curves were used to estimate lag-times and growth rates. Furthermore, the 100 strain set was subdivided into 4 categories, *i.e.* diarrhoeal, emetic, environmental and food strains, to identify potential important contamination routes.
Results: Analysis show a large biodiversity both within and between groups of *B. cereus* strains. Yet, emetic and diarrhoeal strains often form a distinct group with respect to the tested biophysical characteristics in relation to food safety. Emetic strains do not grow at temperatures ≤ 7 °C. Furthermore, their spores are more heat resistant compared to the other groups. This results in a high risk on emesis when preheated food products are being kept outside the fridge. About 75 % of the studied diarrhoeal strains do grow at 7 °C and, although their spores are less heat resistant, they can survive pasteurization processes. Also, growth rates appeared to be optimal at different temperatures in food products with neutral pH.
Recommendations: Statistical data analysis showed that preventing *B. cereus* food borne poisoning in Europe would primarily ask for: 1- fridge temperatures below 7 °C to prevent growth of diarrhoeal strains during storage, 2- minimal growth temperatures of emetic strains (≥ 10 °C) plead for a rapid temperature decrease of precooked food by, for example, portioned chilling in order to prevent growth and, with that, toxin production of emetic strains and 3- producing food products with a *non* neutral pH-value in order to prevent instant growth of bacteria.

1. Introduction

Bacillus cereus is a foodborne pathogen consisting of a huge variety of strains. Its hazard is characterized by the production of both emetic and enterotoxins and survival under extreme conditions due to spore formation (Ehling-Schulz et al., 2004; Granum, 1997; Kotiranta et al., 2000; Wijnands et al., 2002). Still, little is known about the virulence potential in relation to characteristics of specific strains.

Therefore, 100 strains were selected (see Appendix 1) to assess key factors affecting virulence of *B. cereus*. This selection was based on the characterisation of an earlier selected strain set (Ehling-Schulz et al., 2005) and includes strains from the environment, different food products and food poisoning outbreaks. Partners in this project have done several experiments to both qualify and quantify the virulence of different strains. Characterisation of the 100 *B. cereus* strains included:

i. Growth temperatures
ii. Potential toxin production
iii. Heat resistance
iv. Germination
v. Growth characteristics

This report gives a general overview of the experimental results obtained by the different laboratories concerning above mentioned assessments. In addition, parameter estimates were performed using the heat resistance data and provided growth data to extract biologically relevant strain characteristics from the data. To ultimately identify remarkable strain properties in relation to sources, strains were divided into four categories. That is, strains selected from:

1- the *environment* (n=15),
2- *food* products (n=35) and
   strains selected from food poisoning cases, *i.e.*
3- strains shown to have caused emesis (*emetic* strains, n=10) and
4- strains shown to have caused diarrhoea (*diarrhoeal* strains, n=40).

Most of the data and further analysis are presented according to these categories. Results, as found during summarising the data according to *B. cereus* group 1 to 4, were subsequently subjected to statistical analysis. Our goal was to link the results of different bacteriological assessments to strain origin in order to improve hazard identification within the *B. cereus* group. The key question to be answered to accomplish this goal is:

→ Can hazardous strains within the *B. cereus* group be traced based on specific biophysical characteristics?

In order to get insight in what the actual assessments represent and how the experimental results were obtained, a brief overview of the experiments is given in the next section. A short method description and results of the different assessments will be summarised in subsequent separate subsections and remarkable results will be pointed out. Then, an analogous order of subsequent sections will reveal whether remarkable associations/differences between the strain categories are actually supported by statistical analysis. Finally, general conclusions will be followed by recommendations about potential hazards associated with *B. cereus* contamination in Europe.

2. Brief overview of experimental assessments

Table 1 gives a brief overview of the assessments done by the different institutes on the 100 selected *B. cereus* strains. The experiments will be explained in more detail in the separate sections.

Table 1 Laboratories (2nd column) and their assessments (1st column) on the 100 selected Bacillus cereus strains.

Selection of representative strains	Technical University Munich (TUM) and National Institute for Agronomical Research (INRA)
FTIR[1] analysis of strains	TUM
Minimum growth temperature	TUM
Maximum growth temperature	TUM
Effect temperature on growth	TUM
Effect pH on growth	TUM
Emetic toxin production by:	
- PCR-800/NRPS[2] (qualitative, +/- values)	TUM
- Cereulide LC-MS[3] (qualitative, +/- values)	University of Helsinki (UH-DACM)
- Cytotoxicity test (quantitative, titer)	Ludwig Maximilians University (LMU)
Enterotoxin production[4]:	
- Overall (quantitative (titer))	LMU
- HBL-L2 production (quantitative (titer))	LMU
- HBL-B production (quantitative (antigen titer)	LMU
- NHE-A production (qualitative, +/- values (antigen)	LMU
- NHE-B production (quantitative (antigen titer)	LMU
Enterotoxin gene detection[4,5] by:	
- PCR (detecting ≥ 1 gene of the Hbl-operon (qualitative, +/- values)	INRA
- PCR (detecting ≥ 1 gene of the Nhe-operon (qualitative, +/- values by PCR)	INRA
- PCR (cytK gene detection (qualitative, +/- values)	INRA
Heat resistance	INRA
Germination	INRA
RAPD[6]	Swedish Diary Association (SDA)
Adhesion capacity	Norwegian School of Veterinary Science (NVH)

[1]Fourier transformed infrared spectroscopy (Kummerle et al., 1998).
[2]Cereulide is the emetic toxin produced by *B. cereus*. The gene complex responsible for cereulide production (NRPS, Nonribosomal peptide synthetase gene complex) has recently been described by Ehling-Schulz et al. (2005).
[3]Liquid chromatography - mass spectrometry (Haggblom et al., 2002).
[4] *Bacillus cereus* can produce several enterotoxins, three of which are known to cause disease, *i.e.* HBL, NHE and CytK. From the four genes that form the HBL operon, three encode the three enterotoxin proteins, *i.e.* HBL-L2, HBL-L1 and HBL-B. The NHE operon consists of three genes encoding for the enterotoxin proteins NHE-

A, NHE-B and NHE-C. The CytK toxin is produced by expression of the cytK gene. Expression of the HBL, NHE and CytK genes is regulated by the PlcR gene (Okstad et al., 1999; Lund et al., 2000; Granum et al., 1999).
[5](Guinebretiere et al., 2002).
[6]Random amplified polymorphic DNA analysis (Andersson et al., 1999).

Appendix 1 gives a summary of a majority of the assessments as named in Table 1. That is, Appendix 1 includes those assessments that were provided before February 1st, 2004 and used for summarizing purposes and further statistical analysis as described in this report.

Results as presented on the *emetic* strains, however, needs some further explanation. That is, the TUM and UH-DACM did experiments in which 17 of the 100 tested strains were detected to possess the gene related to emetic toxin production. In addition to the 10 food poisoning strains, 5 food strains and 2 environmental strains showed to possess the gene. Results and analysis on “emetic strains” will, therefore, be presented for both the n=17 and n=10 strains according to this argument throughout the report.

3. Experimental results and Data analysis

3.1 Growth temperatures

3.1.1 Experimental results

Table 2 summarizes the results on minimum and maximum growth temperatures of the 100 selected *B. cereus* strains ordered by origin (Environment, Food and Food poisoning). Most notable result is that the strains selected from food poisoning cases show no growth at 4 °C and a majority of these strains have a minimum growth temperature at 7 °C. Detailed information on the behaviour of the food poisoning strains can be seen in Table 3 where food poisoning strains are divided into emetic and diarrhoeal categories. Note that, as explained in Section 2, the emetic category in Table 3 shows both the results on growth temperatures for the strains possessing the emetic toxin gene (n=17, see Appendix 1), and the results for a subgroup of these strains which actually have shown to cause emesis (n=10, see Appendix 1). Yet, all emetic strains seem to need temperatures of ≥ 10 °C to be able to grow. The most frequent minimum growth temperature for diarrhoeal strains seems to be 7 °C. Environmental strains seem to grow less well at temperatures ≥ 48 °C, compared to the other strains.

Table 2 Minimum and maximum growth temperatures of B. cereus strains. Values are expressed in percentages of total strain number (n) per growth temperature according to category.

	Temp.° C	Environment n = 15	Food n = 35	Food poisoning n = 50
min growth temp	4	33	11	0
	7	27	29	58
	10	20	31	26
	>10	20	29	16
max growth temp	≤ 42	13	6	2
	45	60	28	40
	≥ 48	27	66	58

A further characterization on growth temperatures can be based on the division of strains in mesophilic and psychrotolerant categories.

Mesophilic strains are here defined by growth temperatures ≥ 10 °C. Psychrotolerant strains can grow at low temperatures (< 10°C). Table 3 shows that, according to this definition, all emetic strains are mesophilic and a majority of the diarrhoeal strains seem to have psychrotolerant traits.

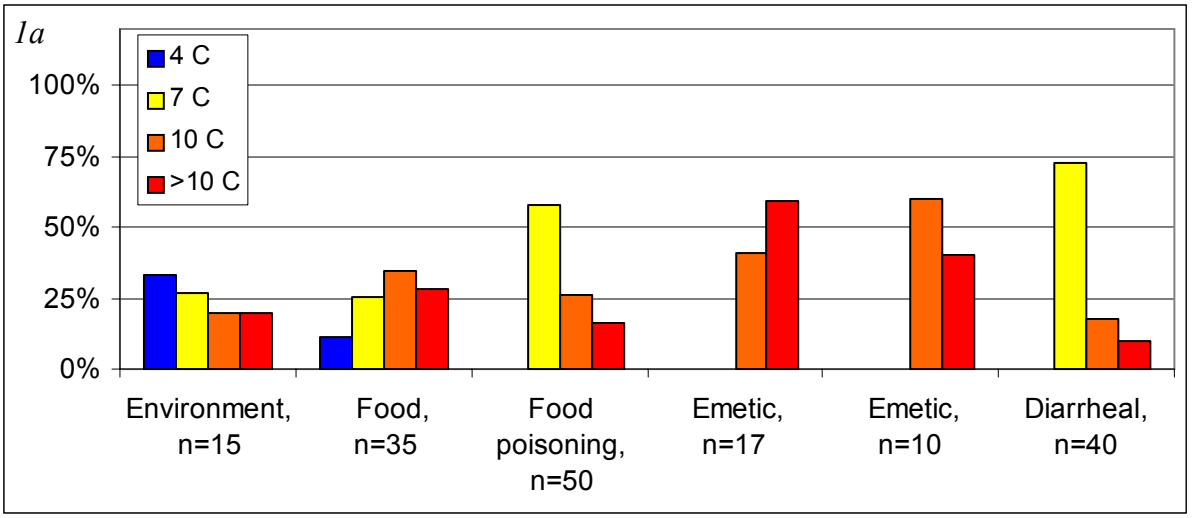
Another method to distinguish psychrotolerant from mesophilic strains is by analysing RAPD patterns (Andersson et al., 1999). However, classification of *B. cereus* in psychrotolerant and mesophilic strains based on RAPD-patterns were not at all consistent with classifications based on growth temperature ranges. Therefore, division of strains based on RAPD patterns were omitted in this research.

Table 3 Minimum and maximum growth temperatures of B. cereus strains specified by the two food poisoning categories. Values are expressed in percentages of total strains (n) per growth temperature according to category.

	Temp. °C	Emetic[1] n = 17 (n=10)	Diarrhoeal n = 40
min growth temp	4	0 (0)	0
	7	0 (0)	72
	10	41 (60)	18
	>10	59 (40)	10
max growth temp	≤42	0 (0)	2
	45	0 (0)	50
	≥48	100 (100)	48

1 Test results using either the n=17 or n=10 emetic strains (see Section 2. for explanation)

A general overview of Tables 2 and 3 on the link between origin of strain and growth temperature ranges is shown in Figure 1a,b. It can immediately be seen that the food poisoning strains used in this study do not grow at 4 °C and, on top of that, strains associated with emesis do not grow at 7 °C. All tested emetic strains have maximum growth temperatures ≥ 48 °C.



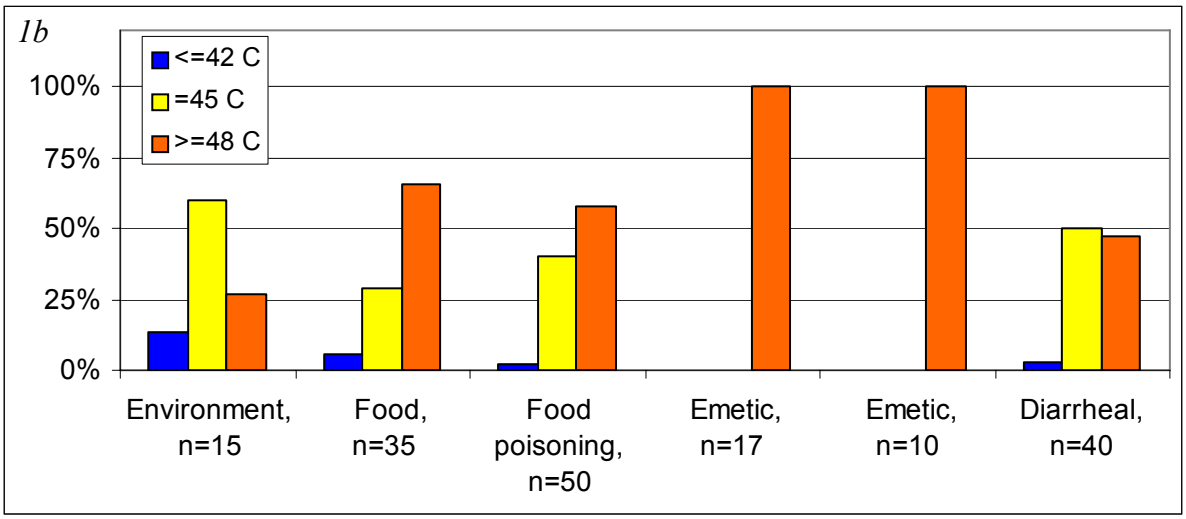


Figure 1a,b Percentages of B. cereus strains in the different growth temperature groups categorized by strain origin. Figure a represents minimum growth temperature groups. Figure b represents the maximum growth temperature groups.

3.1.2 Data analysis

General differences in minimum and maximum growth temperatures between categories of *B. cereus* strains were shown in Table 2. A test for independence (X^2-test, or Goodness-of Fit test) can reveal significant discrepancies in growth temperatures among the categories. This would be useful to get insight in potential hazardous strains for consumers. A first hypothesis is:

- H_0: Proportion of *B.cereus* strains in each temperature group is the same for each category.

In order for a X^2-test to be appropriate, the following assumptions have to be taken into account, *i.e.*:

1- Observed counts are based on a random sample and
2- Sample size is large, that is, every expected count (obtained when the null hypothesis is true) is at least 5.

Since not all expected counts in the minimum growth temperature groups are ≥ 5 , they were combined into two groups, *i.e.* ≤ 7 °C and ≥ 10 °C. Table 4 shows the test results. With a *P*-value < 0.001, H_0 is rejected. There is strong evidence to support the claim that the proportions of *B.cereus* strains which have a minimum growth temperature ≤ 7 °C and ≥ 10 °C is not the same for environmental, food, emetic and diarrhoeal strains. Emetic and diarrhoeal strains have the largest weight in this difference (Table 4). Emetic strains do not seem to grow at low (fridge) temperatures, whereas the number of diarrhoeal strains having low (7 °C) minimum temperatures is higher than the number having minimum temperatures ≥ 10 °C.

Table 4 Observed (Expected) strains per minimum growth temperature group and subsequent results of a X^2-test to reveal differences in minimum growth temperatures of B. cereus strains between strain categories.

	n	≤ 7 °C	≥ 10 °C	X^2	P-value
Environment	15	9 (7)	6 (8)	27	< 0.001
Food	35	14 (17)	21 (18)	df	
Emetic	17[1]	0 (8)	17 (9)	(4-1)(2-1) = 3	
Diarrhoeal	40	29 (19)	11 (21)		

	n	≤ 7 °C	≥ 10 °C	X^2	P-value
Environment	15	9 (8)	6 (7)	20	< 0.001
Food	35	14 (18)	21 (17)	df	
Emetic	10[1]	0 (5)	10 (5)	(4-1)(2-1) = 3	
Diarrhoeal	40	29 (21)	11 (19)		

1 Test results using either the n=17 or n=10 emetic strains (see Section 2. for explanation)

A similar test was performed for maximum growth temperatures (Table 5). The test revealed that the number of strains in the two maximum growth temperature categories are

disproportionally spread over strain categories. More specific, the number of environmental and emetic strains with growth temperatures ≤ 45 °C and ≥ 48 °C are not in the same order as the numbers one would expect when H_0 was true. This implies that environmental strains have relatively low maximum growth temperatures, whereas emetic strains feel comfortable at high temperatures.

Table 5 Observed (Expected) strains per maximum growth temperature group and subsequent results of a X^2-test to reveal differences in maximum growth temperatures of B. cereus strains between strain categories.

	n	≤ 45 °C	≥ 48 °C	X^2	P-value
Environment	15	11(6)	4(9)	20.50	< 0.001
Food	35	13(15)	22(20)	df	
Emetic	17[1]	0(7)	17(10)	(4-1)(2-1) = 3	
Diarrhoeal	40	21(17)	19(23)		

	n	≤ 45 °C	≥ 48 °C	X^2	P-value
Environment	15	11(7)	4(8)	14.83	0.002
Food	35	13(16)	22(19)	df	
Emetic	10[1]	0(5)	10(5)	(4-1)(2-1) = 3	
Diarrhoeal	40	21(18)	19(22)		

[1] Test results using either the n=17 or n=10 emetic strains (see Section 2. for explanation)

Results from the above, general, X^2-tests ask for more elaborated statements about the behaviour of *B. cereus* strains at population level based on the 100 tested strains. Therefore, more statistical analyses were performed on the environmental strains, the diarrhoeal strains and on emetic strains in particular.

First a general hypothesis test concerning minimum growth temperatures of the different strain categories at 7 °C was performed, following:

- H_0: Proportion of *B.cereus* strains that have a minimum growth temperature at 7 °C is the same for each category.

A minimum growth temperature of 7 °C shows to be disproportionately divided among categories (Table 6). Diarrhoeal strains have disproportionately more and emetic strains show to have disproportionately less strains with a minimum growth temperature at 7 °C compared to the other categories. This means storage temperatures for food potentially containing potentially diarrhoeal strains can be very critical.

Another more general question to answer is whether there is a difference between environmental, food, emetic and diarrhoeal strains concerning a maximum growth temperature of ≥ 48 °C. The null hypothesis for this particular question is:

- H_0: Proportion of *B.cereus* strains that have a maximum growth temperature of ≥ 48 °C is the same for each category.

Table 7 shows the distribution of strains per category having a growth temperature ≥ 48 °C and subsequent test results. A *P*-value of <0.001 (0.001 based on 10 emetic strains) shows sufficient evidence to conclude that a relationship exists between a maximum growth temperature of ≥ 48 °C and strain categories. Emetic and environmental strains show to have the highest weight in this difference. Indicating a statistically significant number of environmental strains have a maximum growth temperature which is less than 48 °C and a disproportional large number of emetic strains do grow at maximum temperatures ≥ 48 °C.

Table 6 Observed (Expected) strains per minimum growth temperature group and subsequent results of a X^2-test to reveal differences in minimum growth temperature at 7 °C of B. cereus strains between strain categories.

	7 °C	not at 7 °C	X^2	P-value
Environment	4 (6)	11 (9)	31.90	< 0.001
Food	10 (14)	25 (21)		
Emetic[1]	0 (7)	17 (10)	df	
Diarrhoeal	29 (16)	11 (24)	(4-1)(2-1)=3	
Total	43	64		

	7 °C	not at 7 °C	X^2	P-value
Environment	4 (7)	11 (8)	26.35	< 0.001
Food	10 (15)	25 (20)		
Emetic[1]	0 (4)	10 (6)	df	
Diarrhoeal	29 (17)	11 (23)	(4-1)(2-1)=3	
Total	43	57		

[1] Test results using either the n=17 or n=10 emetic strains (see Section 2. for explanation)

Table 7 Observed (Expected) strains per maximum growth temperature group and subsequent Results of a X^2-test to reveal differences in maximum growth temperature at 48 °C of B. cereus strains between strain categories.

	≥ 48 °C	not ≥ 48 °C	X^2	P-value
Environment	4 (9)	11 (6)	21.12	< 0.001
Food	23 (21)	12 (14)		
Emetic[1]	17 (10)	0 (7)	df	
Diarrhoeal	19 (23)	21 (17)	(4-1)(2-1)=3	
Total	63	44		

	≥ 48 °C	not ≥ 48 °C	X^2	P-value
Environment	4 (8)	11 (7)	15.61	0.001
Food	23 (20)	12 (15)		
Emetic[1]	10 (6)	0 (4)	df	
Diarrhoeal	19 (22)	21 (18)	(4-1)(2-1)=3	
Total	56	44		

[1] Test results using either the n=17 or n=10 emetic strains (see Section 2. for explanation)

Now that we have discovered general features of *B. cereus* strain categories a more in depth analysis concerning properties of individual strain categories can be perfomed.

Investigation of 17 (10) emetic strains on growth characteristics shows that all these strains have a minimum growth temperature ≥ 10 °C and a maximum growth temperature ≥ 48 °C (Table 3). For future hazard identification purposes it would be interesting to know if emetic strains in general have these growth characteristics. Yet, more samples should be tested to be able to draw conclusions on population level about specific growth characteristics of emetic strains. In general, the criteria for calculating a large sample Confidence Interval (C.I.) about a population proportion is:

1- Sample proportion (p) is calculated from a random sample, and
2- Sample size (n) is large (n p ≥ 10 and n(1-p) ≥ 10).

From a hazard characterisation perspective it would, in addition, be interesting to know whether, for example, a majority of the diarrhoeal strains in general have a minimum growth temperature of 7 °C or whether this observation is more likely to be due to sampling variability.

A Goodness-of Fit test can determine whether strains of a specific origin are likely to have equal frequency of minimum growth temperatures at 4, 7 10 and > 10 °C or not. The corresponding hypothesis test is:

- H_0: Proportion of diarrhoeal strains is equal in each minimum growth temperature group.

This test in this particular setting requires a sample size of at least n=20 samples for the expected counts all to be ≥ 5 and so equally distributed over the 4 temperature groups as would be expected under H_0. Considering the tested number of diarrhoeal strains to be n=40, a Goodness-of Fit test was performed with diarrhoeal strains using Table 2. This resulted in a X^2-value of 50.6, with df =3, and so a P-value < 0.001. This means there is sufficient evidence to conclude that a relationship exists between minimum growth temperatures and diarrhoeal strains. Moreover, a significant majority of diarrhoeal strains have a minimum growth temperature at 7 °C. Calculating a C.I. about the proportion of diarrhoeal strains growing at 7 °C can quantify this "significance".
Investigation of the 40 diarrhoeal strains shows that, in fact, 72.5 % of the tested strains were found to grow at a minimum temperature of 7 °C (Table 3). Since n p = 29 and n(1-p) = 11 are both ≥ 10, the sample size is large enough to use the formula for a large-sample confidence interval. Based on this sample data, we can be 95 % confident that the true proportion of diarrhoeal strains that have a minimum growth temperature of 7 °C is between 58.7 and 86.3 %. This would mean that if food is stored at sufficiently low temperatures, *i.e.* < 7 °C, diarrhoeal strains are less likely to multiply.

Only 9 of 100 tested *B.cereus* strains had a minimum growth temperature of 4 °C (*i.e.* 5 environmental and 4 food strains, Table 2). A X^2-test to reveal whether this minimum growth temperature is equally divided over the different strain origins, in a hypothesis test as done above, is therefore redundant. Still it can be noted that non of the 50 (10 emetic, 40 diarrhoeal) food poisoning strains appeared to have a minimum growth temperature at 4 °C. This asks for a more elaborated investigation without going into a hypothesis test for independence.
Assume that the probability of selecting a *B. cereus* strain with a minimum growth temperature of 4 °C is the same for the food poisoning strains (n=50) and the non-food poisoning strains (n=50). Then, the result in the previous paragraph would indicate the probability of selecting a *B. cereus* strain having a minimum growth temperature at 4 °C to be Binomially distributed with a constant probability of successes estimated as 9/50. Following this reasoning, the probability of finding 0 successes out of 50 trials would be Bin(0, 50, 9/50) < 0.001. In other words, the probability of finding 0 out of 50 randomly selected *B. cereus* strains with a minimum growth temperature of 4 °C would be less than 0.1 % under the assumption that growth temperatures for food poisoning strains are not different from non-food poisoning strains. This result suggests that having a growth temperature at 4 °C is strain origin *dependent*. Moreover, it seems like food poisoning strains in general do not grow

at 4 °C. Still, a t-test would need to be performed to show whether a significant number of food poisoning strains do not grow at 4 °C. The hypothesis test is then:

- H_0: Proportion of *B. cereus* strains that grow at 4 °C is the same for non-food poisoning and food poisoning strains.
- H_a: *B. cereus* causing food poisoning has significantly less strains with a minimum growth temperature at 4 °C than the non-food poisoning category has.

A t-test can be performed using the Normal distribution, $N(\mu,\sigma^2)$, as an approximation for the Binomial distribution of minimal growth temperatures. Then, $\mu = n\,\pi$ and $\sigma^2 = n\,\pi\,(1-\pi)$ and the P-value becomes 0.001. Using $\alpha=0.05$, H_0 is rejected and we can conclude that food poisoning *B.cereus* strains have significantly less than 9/50 strains with a minimum growth temperature at 4 °C.

3.2 Toxin production

3.2.1 Experimental results

Emetic toxin production does not seem to be related to strain origin (Table 8). That is, the probability of finding an emetic strain does not seem to be related to whether the strain was selected from the environment, a food product or from a food poisoning case in general. Non of the strains related to diarrhoea outbreaks, however, were emetic toxin producers, nor did they possess the gene. As the emetic strains were preselected from emesis cases (10 strains), or on toxin production (17 strains) it is evident that these strains both have the gene and are toxin producers. As can be seen from Appendix 1, qualitative (+/-) results on the gene responsible for emetic toxin production from TUM (PCR 800/NRPS test, (Ehling-Schulz et al., 2005)) and UH-DACM (Toxicity to sperm cells test, (Haggblom et al., 2002)) were compatible with the quantitative results obtained by LMU (Cytotoxicity test) and, therefore, combined in Table 8.

Table 8 Percentage of B. cereus strains (n) that can produce emetic toxins according to strain origin. Percentages correspond to gene possession.

Origin of strain	n	Emetic toxin producers and gene possession (as % of n)
Environment	15	13
Food	35	14
Food poisoning	50	20
Emetic	17 (10)	100 (,,)
Diarrhoeal	40	0

The previous statement about compatibility of qualitative and quantitative test results for emetic strains does, however, not apply to enterotoxin assessments (Tables 9 to 12). For example, a qualitative positive PCR test for the HBL operon does not indicate a gene is actually expressed in enterotoxin production (Table 11, Appendix 1). This indicates that, for some strains, expression of the HBL genes was not effected by the regulating PlcR gene (see Section 2. for explanation of the mechanism).

Almost all strains produce enterotoxin proteins, *i.e.* either HBL and/or NHE (Table 9 and Appendix 1 “Diarrhoeal (LMU)”). As no tests are available yet to show the production of CytK, Table 10 only shows information about possession of the cytK gene. The cytK gene seems to be related to diarrhoeal strains over strains from other origins (Table 10 and Appendix 1). The Hbl-operon nor HBL-L2/-B production seems to be related to strain origin, except for the emetic strains (Table 11). Almost all of the strains produce some form of NHE enterotoxin and thus possess the gene (Table 12 and Appendix 1).

Table 9 Percentage of B. cereus strains (n) that can produce enterotoxins according to strain origin.

Origin of strain	n	Quantitative overall enterotoxicity test (as % of n)
Environment	15	93
Food	35	89
Food poisoning	50	90
Emetic	17 (10)	82 (80)
Diarrhoeal	40	92

Table 10 Percentage of B. cereus strains (n) that possess the cytK gene according to strain origin.

Origin of strain	n	cytK gene (as % of n)
Environment	15	20
Food	35	37
Food poisoning	50	50
Emetic	17 (10)	12 (20)
Diarrhoeal	40	57

Table 11 Percentage of B. cereus strains (n) that possess at least one of the genes of the Hbl-operon, that can produce HBL-L2 and HBL-B proteins according to strain origin.

Origin of strain	n	Hbl-operon (*i.e.* ≥ 1 gene present) (as % of n)	HBL-L2 production (as % of n)	HBL-B production (as % of n)
Environment	15	87	60	53
Food	35	66	26	26
Food poisoning	50	62	40	38
Emetic	17 (10)	41 (40)	0 („)	0 („)
Diarrhoeal	40	67	50	47

Table 12 Percentage of B. cereus strains (n) that possess at least one of the genes of the Nhe-operon, that can produce NHE-A and NHE-B proteins according to strain origin.

Origin of strain	n	Nhe-operon (*i.e.* ≥ 1 gene present) (as % of n)	NHE-A production (as % of n)	NHE-B production (as % of n)
Environment	15	100	93	93
Food	35	100	91	91
Food poisoning	50	98	98	92
Emetic	17 (10)	100 („)	94 (100)	82 (80)
Diarrhoeal	40	97	97	95

3.2.2 Data analysis

A hypothesis test, using α=0.05, resulted in a difference between the *B.cereus* strains over the categories (Table 12) concerning the cytK gene. The cytK gene has been detected more frequently in diarrhoeal strains and, in general, less frequently in other strains (particularly in

the emetic strains) than would have been expected if proportionality is assumed. Detection of the cytK gene in subsequent strains is, therefore, an indicator for an increased enterotoxic food poisoning hazard.

There is no significant difference in Hbl-operon possession between categories from what would have been expected under the null-hypothesis (Table 14). However, Hbl-L2 and Hbl-B production does differ significantly between strain categories (Tables 15 and 16, respectively). Emetic, followed by environmental, strains have the largest weight in the difference concerning HBL-L2 production. Relatively little emetic strains and many environmental strains produce HBL-L2 compared to the other categories. Emetic, followed by the diarrhoeal, strains show to have the largest impact on the X^2-value for HBL-B production. Again, relatively little emetic strains, but, in this case, many diarrhoeal strains produce HBL-B compared to the other categories. This might indicate that one should rather select on HBL-B production than on HBL-L2 production when looking for hazardous strains. And, gene possession does not seem to reveal any information on strain origin concerning the HBL enterotoxin.

Table 13 Observed (expected) cytK gene possession per group and subsequent results of a X^2-test to reveal differences in cytK gene possession of B. cereus strains between strain categories.

	n	cytK gene	no cytK gene	X^2	P-value
Environment	15	3 (6)	12 (9)	13.45	0.004
Food	35	13 (13)	22 (22)	Df	
Emetic[1]	17	2 (7)	15 (10)	(4-1)(2-1) = 3	
Diarrhoeal	40	23 (15)	17 (25)		

	n	cytK gene	no cytK gene	X^2	P-value
Environment	15	3 (6)	12 (9)	9.27	0.026
Food	35	13 (14)	22 (21)	Df	
Emetic[1]	10	2 (4)	8 (6)	(4-1)(2-1) = 3	
Diarrhoeal	40	23 (17)	17 (23)		

[1] Test results using either the n=17 or n=10 emetic strains (see Section 2. for explanation)

Table 14 Observed (expected)Hbl-operon possession per group and subsequent results of a X^2-test to reveal differences in Hbl-operon possession of B. cereus strains between strain categories.

	n	Hbl-operon	no Hbl-operon	X^2	P-value
Environment	15	13 (10)	2 (5)	7.49	0.058
Food	35	23 (23)	12 (12)	Df	
Emetic[1]	17	7 (11)	10 (6)	(4-1)(2-1) = 3	
Diarrhoeal	40	27 (26)	13 (14)		

	n	Hbl-operon	no Hbl-operon	X^2	P-value
Environment	15	13 (10)	2 (5)	5.95	0.114
Food	35	23 (23)	12 (12)	Df	
Emetic[1]	10	4 (7)	16 (3)	(4-1)(2-1) = 3	
Diarrhoeal	40	27 (27)	13 (13)		

[1] Test results using either the n=17 or n=10 emetic strains (see Section 2. for explanation)

Table 15 Observed (expected) HBL-L2 production per group and subsequent results of a X^2-test to reveal differences in HBL-L2 production of B. cereus strains between strain categories.

	n	HBL-L2	no HBL-L2	X^2	P-value
Environment	15	9 (5)	6 (10)	18.4	<0.001
Food	35	9 (13)	26 (22)	Df	
Emetic[1]	17	0 (6)	17 (11)	(4-1)(2-1) = 3	
Diarrhoeal	40	20 (14)	20 (26)		

	n	HBL-L2	no HBL-L2	X^2	P-value
Environment	15	9 (6)	6 (9)	13.90	0.003
Food	35	9 (13)	26 (22)	Df	
Emetic[1]	10	0 (4)	10 (6)	(4-1)(2-1) = 3	
Diarrhoeal	40	20 (15)	20 (25)		

[1] Test results using either the n=17 or n=10 emetic strains (see Section 2. for explanation)

Table 16 Observed (expected) HBL-B production per group and subsequent results of a X^2-test to reveal differences in HBL-B production of B. cereus strains between strain categories.

	n	HBL-B	no HBL-B	X^2	P-value
Environment	15	8 (5)	7 (10)	15.6	0.001
Food	35	9 (12)	26 (23)	Df	
Emetic[1]	17	0 (6)	17 (11)	(4-1)(2-1) = 3	
Diarrhoeal	40	19 (13)	21 (27)		

	n	HBL-B	no HBL-B	X^2	P-value
Environment	15	8 (5)	7 (10)	11.5	0.009
Food	35	9 (13)	26 (22)	Df	
Emetic[1]	10	0 (4)	10 (6)	(4-1)(2-1) = 3	
Diarrhoeal	40	19 (14)	21 (26)		

[1] Test results using either the n=17 or n=10 emetic strains (see Section 2. for explanation)

3.3 Heat resistance

3.3.1 Experimental results

Decimal reduction times (*D*-values) were calculated from heat resistance experiments (INRA, Appendix 1) in which the number of cultivable spores (*S*) were assessed after 0, 10, 20, 30, 60 and 120 minutes at 90 °C (Carlin et al., submitted). *D*-values were estimated using a least squares fit of the data to the linear model (Van Gerwen and Zwietering, 1998)

$$\ln(S_t) = \ln(S_0) - k\,\mathrm{t}\,, \qquad (1)$$

where,
k = ln(10) / D,
S_0 = number of spores at t=0.

Spores of four strains could not be counted at a certain time. In those cases a regression line, and so a *D*-value, was based on counts at the remaining five points in time. For two strains (one environmental and one food strain) no spores were detected on the agar plates during the experiments, which were evidently left out of the calculations.

Estimates for both the arithmetic mean ($\bar{x}$) and the median (m) *D*-values for *B. cereus* according to strain origin are shown in Table 17. The fact that all estimated median *D*-values lie below the mean indicates *D*-values for the 98 selected strains are positively skewed

(Figure 2). Emetic strains seem to have a relatively high inactivation time, *i.e.* slow inactivation. Furthermore, the 5 emetic strains with very slow spore inactivation (*i.e.* *D*-values ≥ 500 min., Figure 2) all stem from the strains that have shown to cause emesis (n=10). Still, one should consider that these estimates lie well outside the time range of the experiments which ran up to 120 min. and so these values should be interpreted with caution.

Table 17 Estimated D-values (decimal reduction times), using eq. 1, per B. cereus strain category (n). Values represent averages ($\bar{x}$) and median (m) values.

Origin of strain	n	*D*-value, $\bar{x}$ (min)	*D*-value, m (min)
Environment	14	109	85
Food	34	193	140
Food poisoning	50	197	92
Emetic	17 (10)	424 (496)	371 (455)
Diarrhoeal	40	123	72

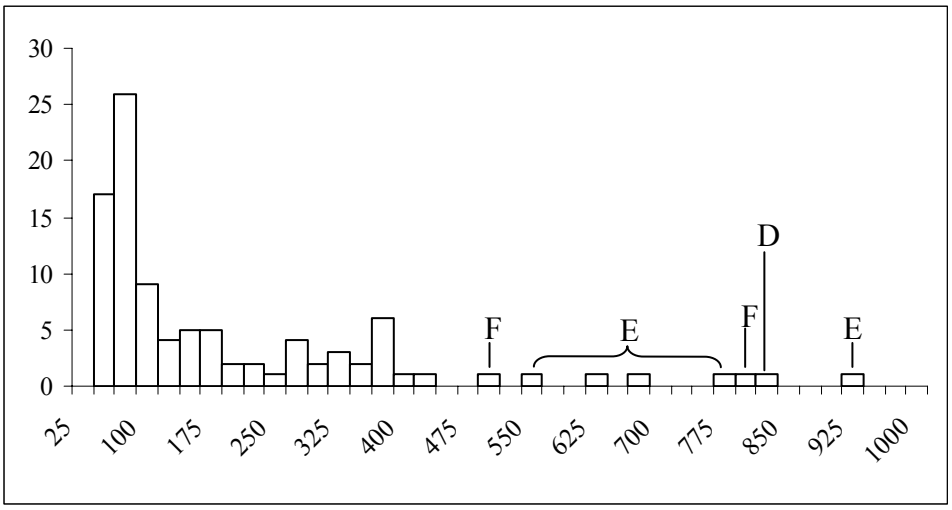


Figure 2 Distribution of D-values (decimal reduction times) for 98 B. cereus strains. D-values ≥ 500 min. (over 8 hours) consist of 5 emetic strains (E), 2 food strains (F) and 1 diarrhoeal strain (D).

In addition, inactivation data often show a non-linear relationship. Instead of using a *D*-value to quantify spore inactivation, another statistic, *i.e.* time until the first decimal reduction (*d*-value, (Mafart et al., 2002) can be estimated using a least squares fit of the data to the non-linear Weibull model

$$\mathrm{Log}(S_t) = \mathrm{Log}(S_0) - \left(\frac{\mathrm{t}}{d}\right)^p, \qquad (2)$$

where
p = shape parameter.

Results of the average, $\bar{x}$, and median, m, *d*-values per category are given in Table 18. Also *d*-values show higher average values than median values, indicating positive skewness of this variable (Figure 3). The emetic strains seem to have higher *d*-values. In addition to the extreme *d*-value for one emetic and a food strain (Figure 3), more extreme values were found for two emetic strains, a food strain and a diarrhoeal strain, with estimated *d*-values of 1007, 1145, 4309 and 6266 minutes, respectively (not shown). Because these few large *d*-values increase the average, it would be better to use the median to quantify the center of this distribution. It seems like strains selected from the environment are more sensitive to heat, with both a low mean and a low median *d*-value, compared to other strains (Table 18). Furthermore, emetic strains seem to be highly heat resistant.

Table 18 Estimated d-values (time until first decimal reduction), using eq.2, per B. cereus strain category (n). Values represent averages ($\bar{x}$) and median (m) values.

Origin of strain	n	*d*-value, $\bar{x}$ (min)	*d*-value, m (min)
Environment	14	37	12
Food	34	179	24
Food poisoning	50	215	31
Emetic	17 (10)	241 (329)	130 (137)
Diarrhoeal	40	187	25

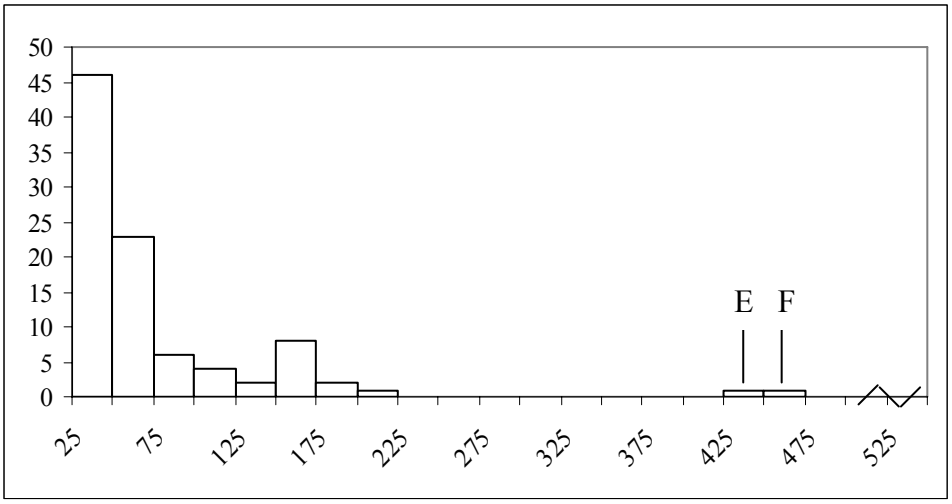


Figure 3 Distribution of d-values (time until first decimal reduction) for 98 B. cereus strains. d-values ≥ 425 min (over 7 hours) consist of at least 1 emetic (E) and 1 food (F) strain. A further 4 "extreme" values are not shown here.

Figures 4 and 5 show examples of fitting both the linear (eq.1) and non-linear (eq.2) model to data of a food poisoning (diarrhoeal, WSBC 10605 (Appendix 1)) strain (D=49, d=15.6, p=0.86) and a strain originating from food (WSBC 10616 (Appendix 1) (D=48, d=1.87, p=0.46) respectively. It seems like a non-linear model (*i.e.*, where p > 1 or p < 1) does not always improve the fit (Figure 4). Still, Table 11 shows that, on average, p values seem to differ from 1 and so a non-linear model would in most cases be preferred over a linear model to fit the data. In addition, the median p-values show that 50 % of all p-values are smaller than 0.3, 0.5 or 0.6. This means that at least 50 % of all inactivation curves show a non-linear concave shape as shown in Figure 5.

Table 19 Estimated p-values (shape parameter), using eq.3, per B. cereus strain category (n). Values represent averages ($\bar{x}$) and median (m) values.

Origin of strain	n	p-value, $\bar{x}$	p-value, m
Environment	14	0.7	0.6
Food	34	0.5	0.5
Food poisoning	50	1.4	0.6
Emetic	17 (10)	2.1 (3.4)	0.3 (,,)
Diarrhoeal	40	0.9	0.6

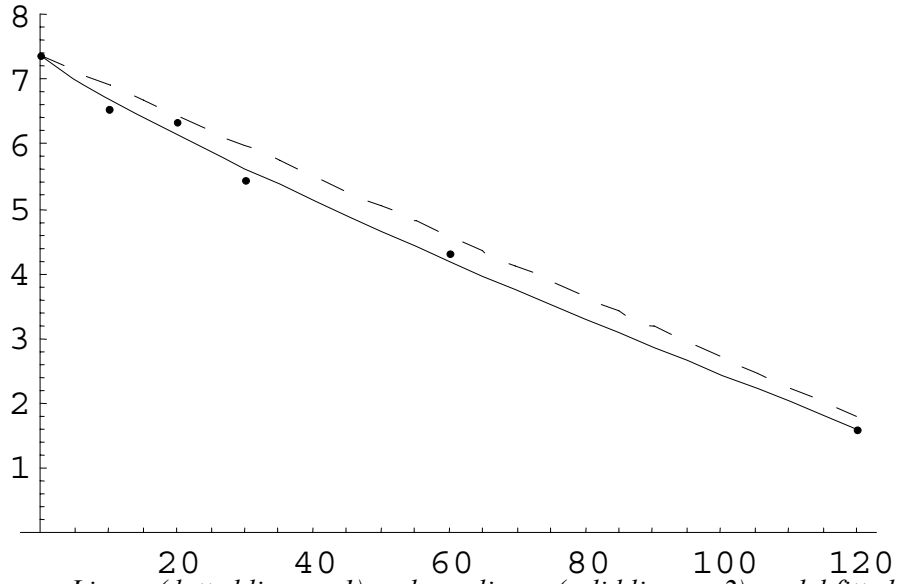


Figure 4 Linear (dotted line, eq.1) and non-linear (solid line, eq.2) model fitted to inactivation data of a B. cereus strain selected from a diarrhoeal source (D=49, d=15.6, p=0.86).

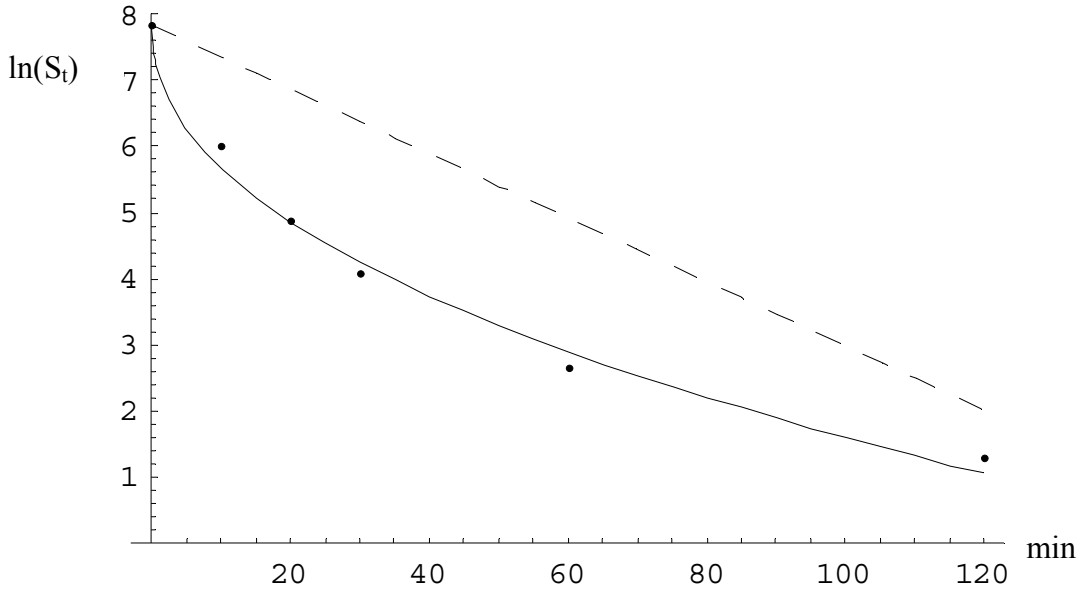


Figure 5 Linear (dotted line, eq.1) and non-linear (solid line, eq.2) model fitted to inactivation data of a B. cereus strain selected from a food source (D=48, d=1.87, p=0.46)

3.3.2 Data analysis

Experiments on heat resistance showed relatively high decimal reduction times (*D*-values) for the selected emetic strains (Table 17). A test to show whether emetic strains give statistically higher average *D*-values seems unreasonable as only 17 (10) emetic strains were used. As n is not ≥ 30, a test would only be valid if a Normal distribution of *D*-values would be assumed for emetic strains on population level. Still, without going into detailed tests, 95 % confidence intervals (C.I.'s) about the mean were calculated for *B. cereus* strains according to

category (Table 20). This gives a general indication of the spread of *D*-values about the mean. Any further statistical inferences concerning emetic and environmental strains will be omitted due to the small sample size (n<30).

Table 20 95 % C.I.'s (lower value, Min, and upper value, Max) and average decimal reduction times (D-value) of B. cereus strains according to strain category.

	n	Min.	Average	Max.
Environment	14	48	109	170
Food	34	134	193	252
Emetic	17	311	424	537
Emetic	10	320	496	672
Diarrhoeal	40	79	123	167

Figure 6 visualizes the C.I.'s of Table 20. Although more emetic strains should be tested, these results give an indication that emetic strains have higher *D*-values. That is, the C.I. for emetic strains does not overlap with the C.I. for the other strains (Table 20 and Figure 6). This would indicate that if a relatively high *D*-value was to be estimated for a future isolated *B.cereus* strain one could conclude it very likely to be an emetic strain. Meaning, inactivation during heating would be slow. As all other C.I.'s show some overlap between categories, no further indications for possible strain properties could be given based on these results.

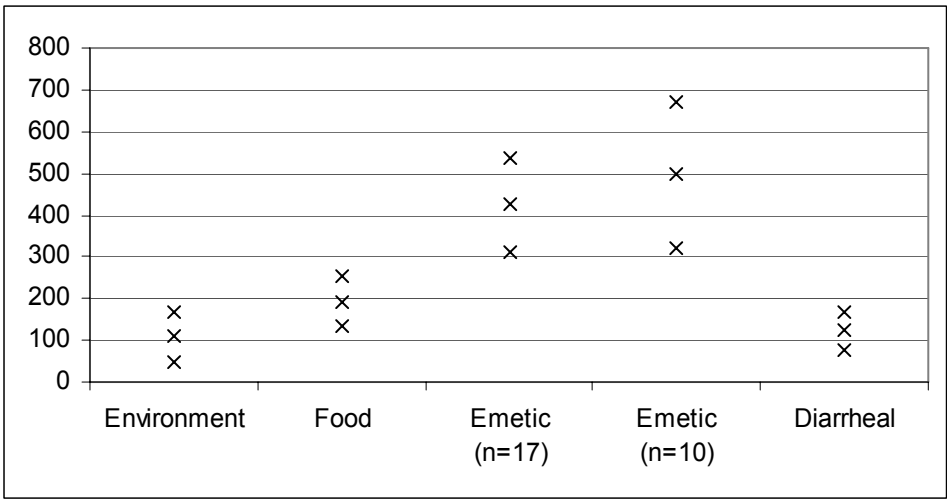


Figure 6 95 % C.I.'s (lower value (Min.) and upper value (Max.)) and average decimal reduction times (D-value) of B. cereus strains according to strain category

Note, however, that C.I.'s for the environmental and emetic strains are only valid under a Normal population distribution of *D*-values. In addition, recall that 42 of the estimated *D*-values lie outside the time range of the experiment, *i.e.* > 120 minutes (see Appendix 1). One should, therefore, be cautious in drawing conclusions from these estimates. That is, bringing *D*-value estimates of over 8 hours into practice based on experimental inactivation data of 2 hours should be avoided. Still, these estimates can very well be used for qualitative comparison reasons. Furthermore, weighted average *D*-values should be calculated when averages are based on individual parameter estimates. That is, each estimated *D*-value (per strain) has a standard error. One should account for these individual standard errors when calculating an average *D*-value and resulting C.I. per category. As for the explorative character of this study the use of weighted averages was omitted in any of the analysis in this section.

Table 18 showed that emetic strains seem to have higher *d*-values and environmental strains to have lower *d*-values compared to the other categories. However, when a C.I. is calculated for the average *d*-value per category, these indications vanish (Table 21 and Figure 7). Note that the large variability in the data causes large C.I.'s about the mean resulting in the negative minimum values in Table 21. Although Tables 17 and 18 could imply that the high *D*-values for emetic strains are associated with high *d*-values, correlation is moderate (r=0.63).

Table 21 95 % C.I.'s (lower value, Min., and upper value, Max.) and average time until first decimal reduction (d-value) of B. cereus strains according to strain category.

	n	Min.	Average	Max.
Environment	14	5	37	69
Food	34	-77	179	435
Emetic	17	66	241	416
Emetic	10	36	329	622
Diarrhoeal	40	-128	187	502

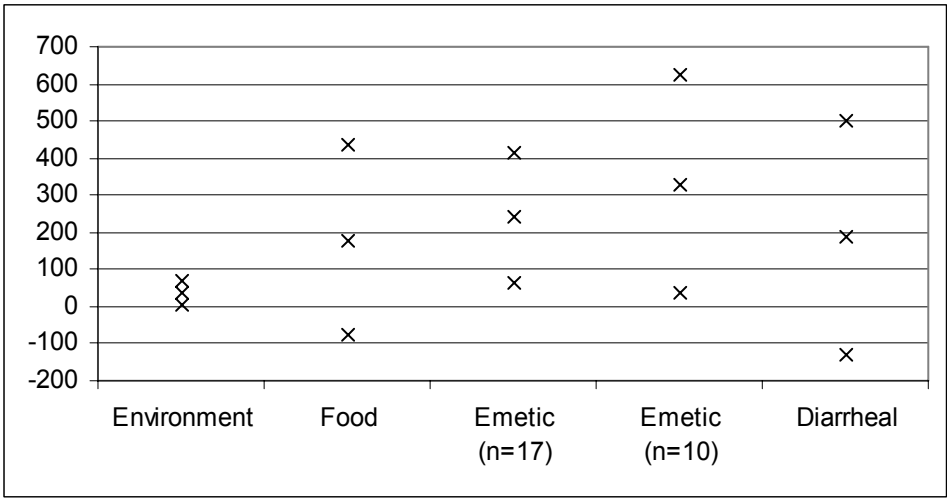


Figure 7 95 % C.I.'s (lower value, Min., and upper value, Max.) and average time until first decimal reduction (d-value) of B. cereus strains according to strain category.

The value of the shape parameter *p* of the non-linear model seemed, with an average value of 2.15 (3.44), to result in more convex inactivation curves for emetic strains compared to the other categories (Table 22). However, this result is not strengthened when comparing the C.I.'s about the mean for the shape parameter between categories (Table 22 and Figure 8). Again, note that the negative minimum values in Table 22 are a result of the large variability in the data. This, subsequently, causes large C.I.'s about the mean.

Table 22 95 % C.I.'s (lower value, Min., and upper value, Max.) and average value of the shape parameter in eq.2 (p-value) of B. cereus strains according to strain category.

	n	Min.	Average	Max.
Environment	14	0.47	0.68	0.89
Food	34	0.42	0.55	0.68
Emetic	17	-0.44	2.15	4.74
Emetic	10	-1.12	3.44	8
Diarrhoeal	40	0.53	0.89	1.25

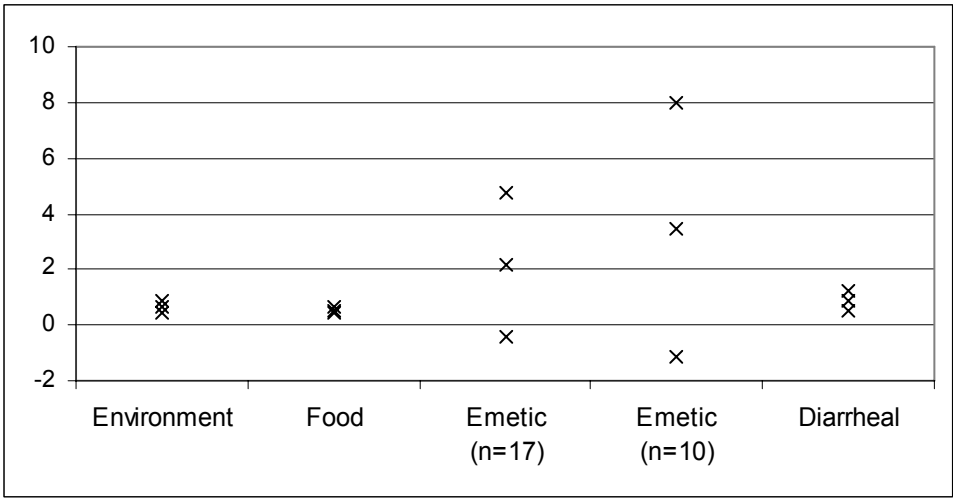


Figure 8 95 % C.I.'s (lower value, Min., and upper value, Max.) and average value of the shape parameter in eq.2 (p-value) of B. cereus strains according to strain category.

In addition, a *t*-test was performed to analyse whether the average value for the shape parameter differs significantly from 1 in the different categories. In other words, can a non-linear model, on average, better predict the inactivation for future strains. Table 23 shows the resulting P-values, indicating that, except for the emetic strains, with p=2.15 (P-value=0.1730) and p =3.44 (P-value=0.113), and the diarrhoeal strains, with p =0.89 (P-value=0.2683), the average values for the shape parameter p differ significantly from 1. Note, however, that, when accounting for sample sizes, this conclusion is only valid for the food and the diarrhoeal strains. More specific, food strains have, on average, non-linear concave shape inactivation curves as shown in Figure 5. Meaning, relatively fast inactivation during heating. Inactivation of diarrhoeal strains can, on average, equally well be described by the more simple linear model.
Estimated p-values for the individual strains shows that a majority (≥ 70 %) of *B.cereus* strains have concave shaped inactivation curves (*i.e.* $p < 1$) independent of strain category (Table 24).

Table 23 Results of a t-test revealing whether the average estimates of the p-values in Table 24 differ significantly from 1, i.e. P(p<1) or P(p>1).

Environment	14	P(p<1) = < 0.001
Food	34	P(p<1) = < 0.001
Emetic	17	P(p>1) = 0.173
Emetic	10	P(p>1) = 0.113
Diarrhoeal	40	P(p<1) = 0.268

Table 24 Percentage of strains (n) with concave shaped inactivation curves (p<1) according to strain category.

Emetic	n=17	82 %
Emetic	10	70 %
Environment	14	71 %
Food	34	85 %
Diarrhoeal	40	83 %

3.4 Germination

3.4.1 Experimental results

Bacillus cereus spores of the 100 strain set were incubated at 7 °C for 7 days and at 30 °C for 50 minutes (INRA). Spore numbers were counted just before incubation and after pasteurization at the end of the incubation period. Replicates were performed for each experiment. The extent of germination was expressed as

$$x = {}^{10}Log\left(\frac{N_0}{N_1}\right), \tag{3}$$

where

N_0 Initial spore number,

N_1 Number of spores after incubation.

Average germination values were calculated from the replicates for each strain (Appendix 1). Subsequently, an average ($\bar{x}$) and median (m) germination value was calculated for each category of strain origin (Table 25). As germination is expressed as ${}^{10}Log(N_0 / N_1)$, resulting values should be exceeding 0 for those strains where germination occurred. And, high values means many spores have germinated. The actual percentage of germination can easily be derived from equation 3, where:

$$N_0 = 10^x N_1 . \tag{4}$$

Substituting the fraction of spores germinated,

$$\frac{N_0 - N_1}{N_0}, \tag{5}$$

in eq. 4 gives the

$$\text{fraction germinated} = 1 - 10^{-x} \quad (6)$$

as a readily usable formula to calculate germination percentages. For example, the number 1.8 in Table 25 indicates that, on average, 98 % of spores were germinated after an incubation period of 7 days at 7 °C.

Table 25 Germination of B. cereus strains at 7 °C and 30 °C according to strain category (n). Values are expressed in average ($\bar{x}$) and median (m) values following eq.3.

Origin of strain	n	7 °C, $\bar{x}$	30 °C, $\bar{x}$	7 °C, m	30 °C, m
Environment	14	1.80	2.06	2.07	1.89
Food	34	1.53	1.90	1.61	1.80
Food poisoning	50	1.49	2.50	1.47	2.36
Emetic	17 (10)	0.55 (0.38)	1.66 (1.64)	0.25 (0.23)	1.79 (1.85)
Diarrhoeal	40	1.77	2.72	1.78	2.56

Table 25 shows that emetic strains have the lowest average germination value, both at 7 °C and at 30 °C, corresponding to percentages of 72 (58) and 98 (98) for the 17 (10) selected strains respectively. Environmental strains show to have the highest average germination at 7 °C (98 %) and diarrhoeal strains at 30 °C (100 %). Still, recall that germination values are expressed on a logarithmic scale and a value > 1 in Table 25 indicates a germination percentage > 90 %. This means that all but the emetic strains at 7 °C show high germination.

Figures 9 to 12 show the distribution of the germination values at 7 °C and 30 °C for the strains in the different categories. These figures confirm the observations from Table 25. Figures 9 and 11 show the difference in spore germination between the food poisoning strains in particular. Diarrhoeal strains seem to germinate better both at 7 °C and at 30 °C than emetic strains do. Furthermore, Figures 9 to 12 seem to show a large amount of variability in germination values of strains within the same category. Again, notice that a value of 1 corresponds to germination of 90 % and a value of 5 indicates > 99 % of the spores have germinated.

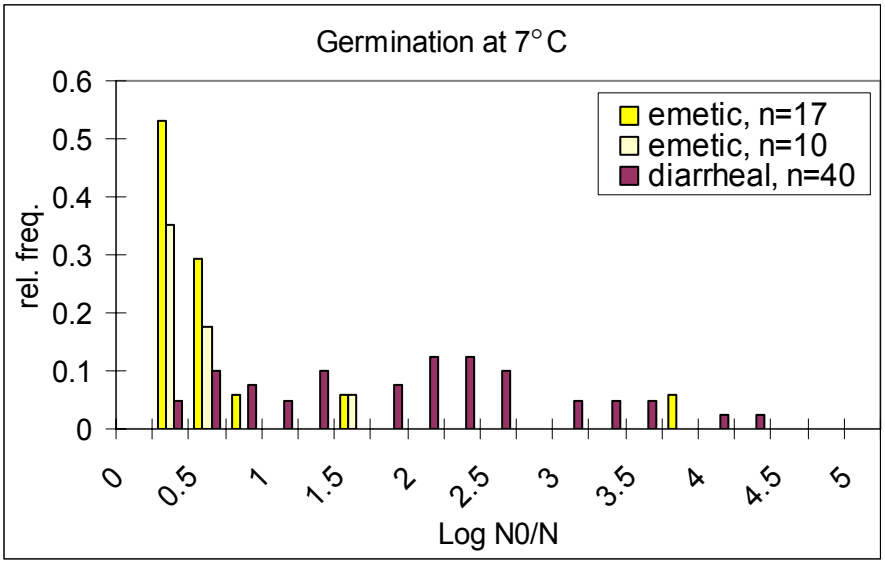


Figure 9 Distribution of germination values of B. cereus strains (at 7 °C) selected from emetic and diarrhoeal food poisoning cases.

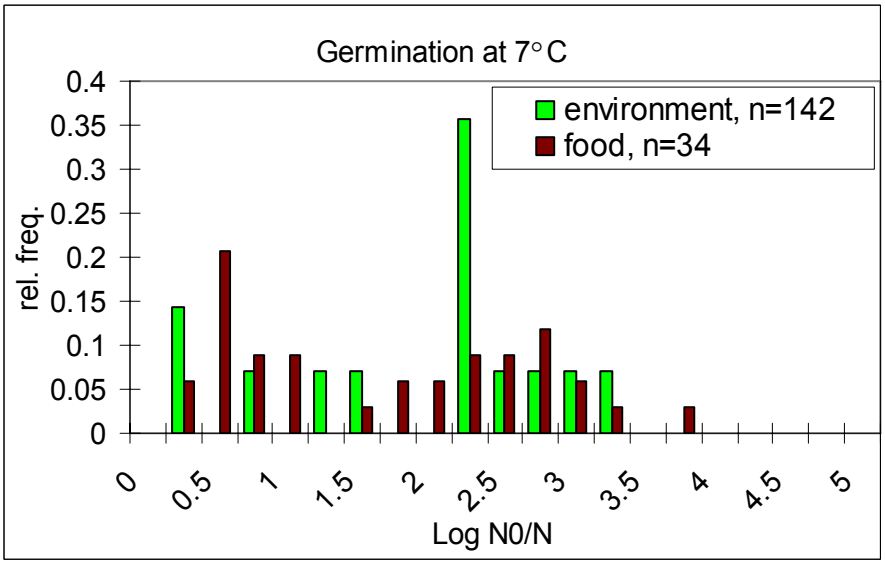


Figure 10 Distribution of germination values of B. cereus strains (at 7 °C) selected from the environment and food products.

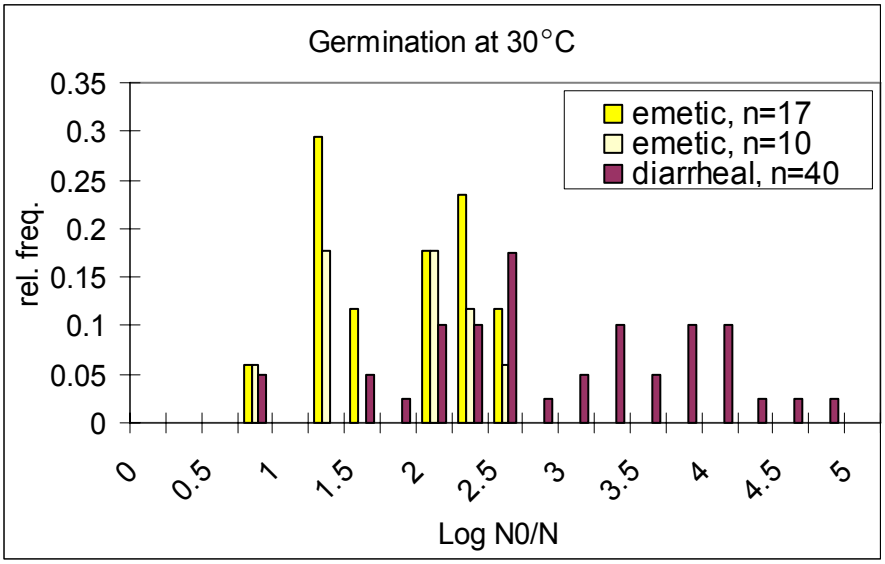


Figure 11 Distribution of germination values of B. cereus strains (at 30 °C) selected from emetic and diarrhoeal food poisoning cases.

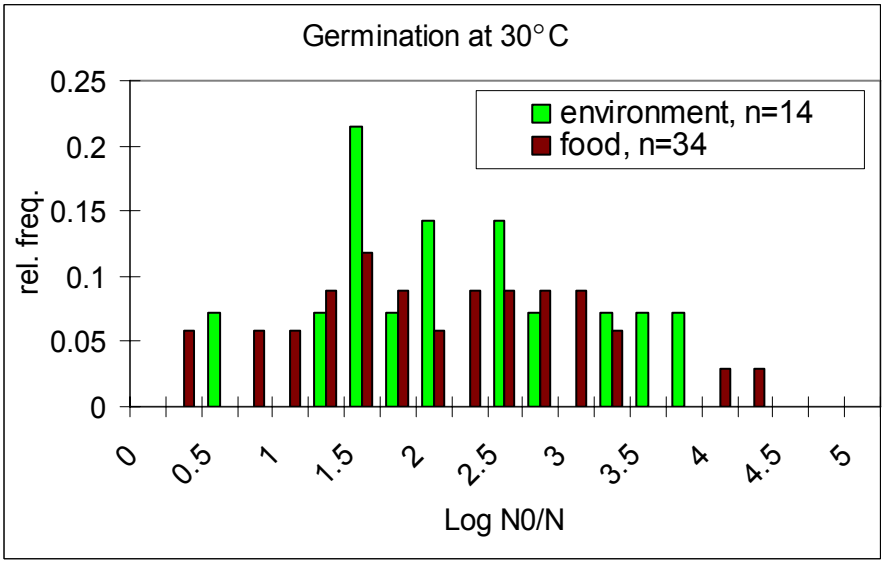


Figure 12 Distribution of germination values of B. cereus strains (at 30 °C) selected from the environment and food products

3.4.2 Data analysis

Possible differences between *B. cereus* strains based on calculated germination values were pointed out in section 3.4.1. Further investigations on the average germination potential between categories of *B. cereus* strains are shown in Table 26 and Figure 13.
For the same reason as in the previous section only 95 % confidence intervals were calculated to point out potential differences between strains of different categories. Table 26 shows a relatively low average germination value for emetic strains at 7 °C. Figure 13 confirms this

observation when comparing C.I.'s for the different categories. Environmental strains, however, do not seem to give higher germination values compared to other strains at 7 °C.

Table 26 95 % C.I.'s (lower value, Min., and upper value, Max.) and average germination value of B. cereus strains at 7 and (30) °C according to strain category.

	n	Min.	Average	Max.
Environment	14	1.26 (1.55)	1.80 (2.06)	2.34 (2.57)
Food	34	1.17 (1.56)	1.53 (1.90)	1.89 (2.24)
Emetic	17	0.10 (1.39)	0.55 (1.66)	1.00 (1.93)
Emetic	10	0.13 (1.25)	0.38 (1.64)	0.63 (2.03)
Diarrhoeal	40	1.43 (2.41)	1.77 (2.72)	2.11 (3.03)

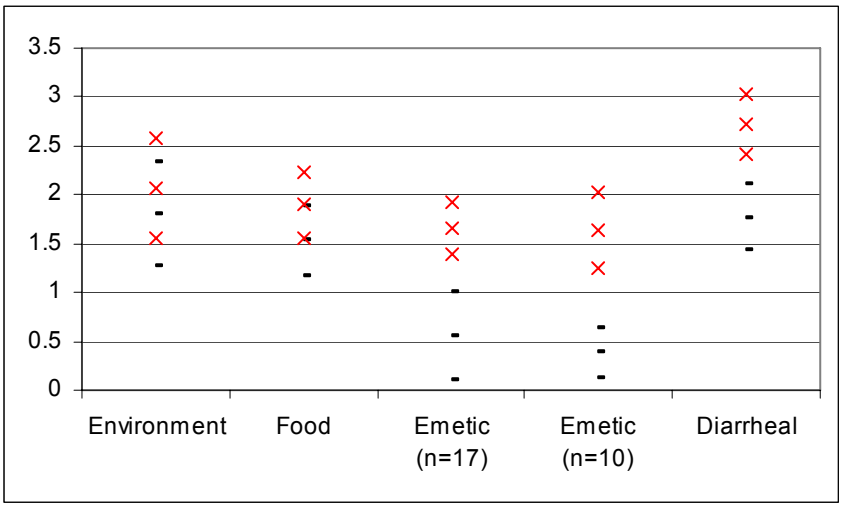


Figure 13 95 % C.I.'s (lower value, Min., and upper value, Max.) and average germination value of B. cereus strains at 7 °C (solid lines) and 30 °C (crosses) according to strain category.

Although diarrhoeal strains do seem to show little overlap with environmental strains for germination values at 30 °C, finding a relatively high germination value would not necessarily indicate the selection of a *B. cereus* strain that causes diarrhoea. That is, a germination value of > 1 on this scale means a germination percentage of > 90 % (see Section 3.4.1). Furthermore, although emetic strains have the lowest average germination value at 30 °C, this observation is not significant when comparing C.I.'s for the different categories (Figure 13)

3.5 Growth characteristics

3.5.1 Experimental results

3.5.1.1 Model fitting

Bacterial growth was assessed experimentally using an optical density (OD) method (TUM). Selected *B. cereus* strains were suspended in a liquid medium (buffered PC broth) and the proportion of light absorbed by the culture in time (also called the optical density) was measured using a photometer. The relationship between OD and cell density is

$OD = -xcl$, where (7)

x is a scaling constant
c is concentration of cells
l is length of light path.

This linear relationship is, however, only valid over an OD range from 0.01 to 0.14, which corresponds to cell densities in the order of 10^6 to $10^{7.5}$ cells ml^{-1} (McMeekin et al., 1993)

The influence of temperature and pH on bacterial growth (expressed in OD values) over time was tested using two temperatures, *i.e.* 24 ^{0}C and 37 ^{0}C, and three pH values, *i.e.* pH 5, 7 and 8. With these temperature and pH combinations *B. cereus* growth was assessed in a photometer during 48 hour experiments. The OD value was measured automatically every hour by the photometer. As experiments for each of the 100 strains was done in at least three fold, this resulted in at least 1800 growth curves, *i.e.* 2 temperatures * 3 pH values * 100 strains * ≥ 3 experiments per strain (see Table 27 in Section 3.5.1.2).

Experimental results were used to quantify growth characteristics for the 100 selected *B. cereus* strains. For this purpose the growth model developed by Baranyi and Roberts (1994) was fitted to the OD data, and the four parameters growth rate (expressed as the change in the optical density per time unit, OD h^{-1}), lag time (h), minimum OD (OD_0) and maximum OD (OD_{max}) were estimated. Figures 14 and 15 show some common (left hand side) and some atypical fits (right hand side) of the model to the data.

Reliable growth characteristics can generally be measured within the first 24 hours of an in vitro culture. After that, lack of nutrients or turbid medium can influence the measurements. Therefore, fitted curves are based on the first 24 hours in the experiment.

In general, the Baranyi and Roberts (1994) model fits the data well, except for measurements done at 37 °C and pH 8 (Figure 15 bottom left hand side). At 37 °C, pH 8, the culture

reaches its stationary phase already within the first 10 hours of the experiment, followed by a decrease in OD value. As the model gives equal weight to all measurements over the 24 hour period, a negative growth rate is estimated. As these fits do not represent a realistic growth pattern, the model was again fitted to the data now only including the first 10 hours of the measurements (Figure 16). This figure shows improved fits and, in addition, in cases where OD values dropped within 10 hours, these values did not result in a negative growth rate estimate.

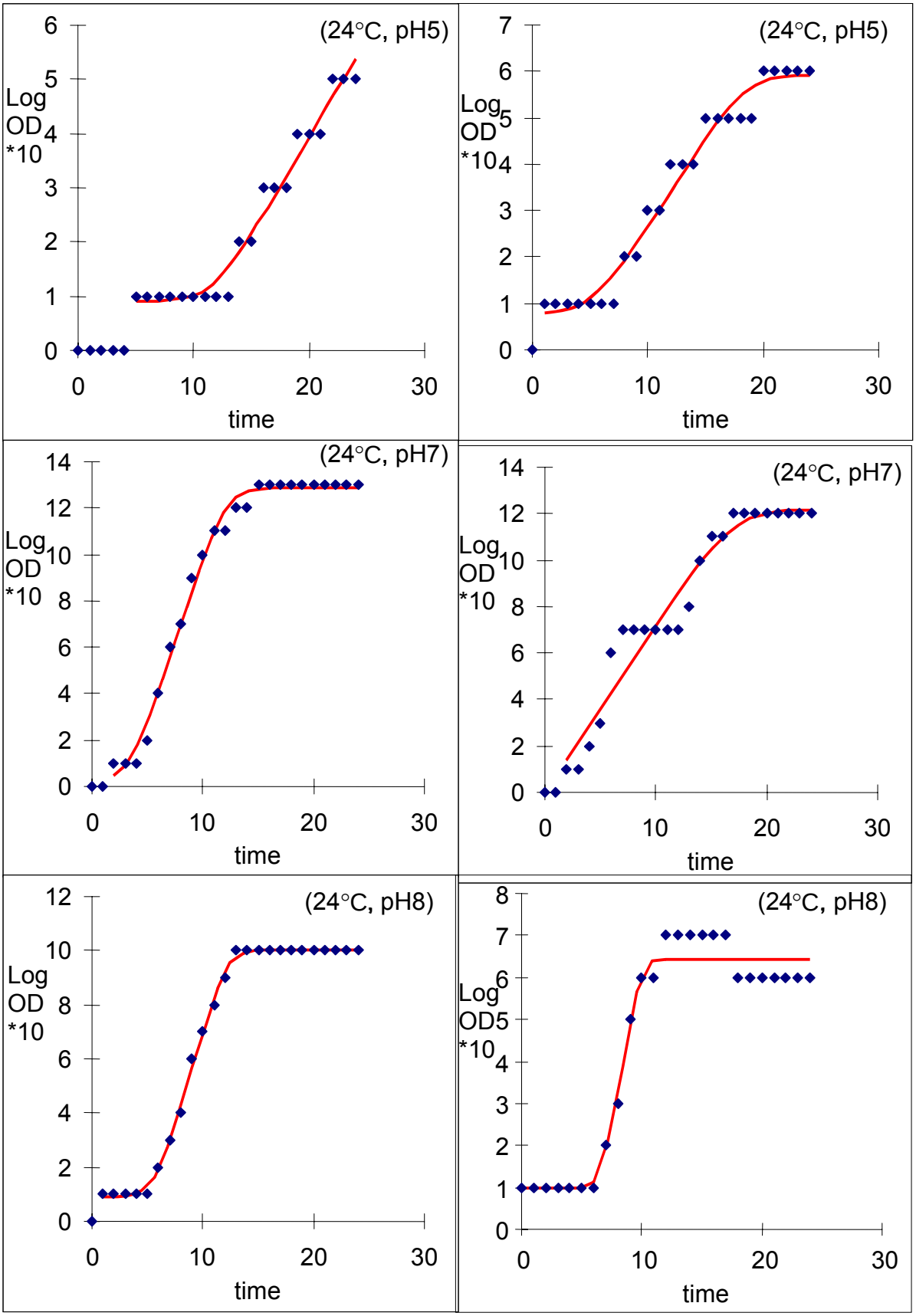


Figure 14 Common (left column) and atypical (right column) growth curves fitted with the Baranyi and Roberts (1994) model to data of B. cereus growth at 24 °C and pH 5 (top row), pH 7 (middle row) and pH 8 (bottom row).

°C,

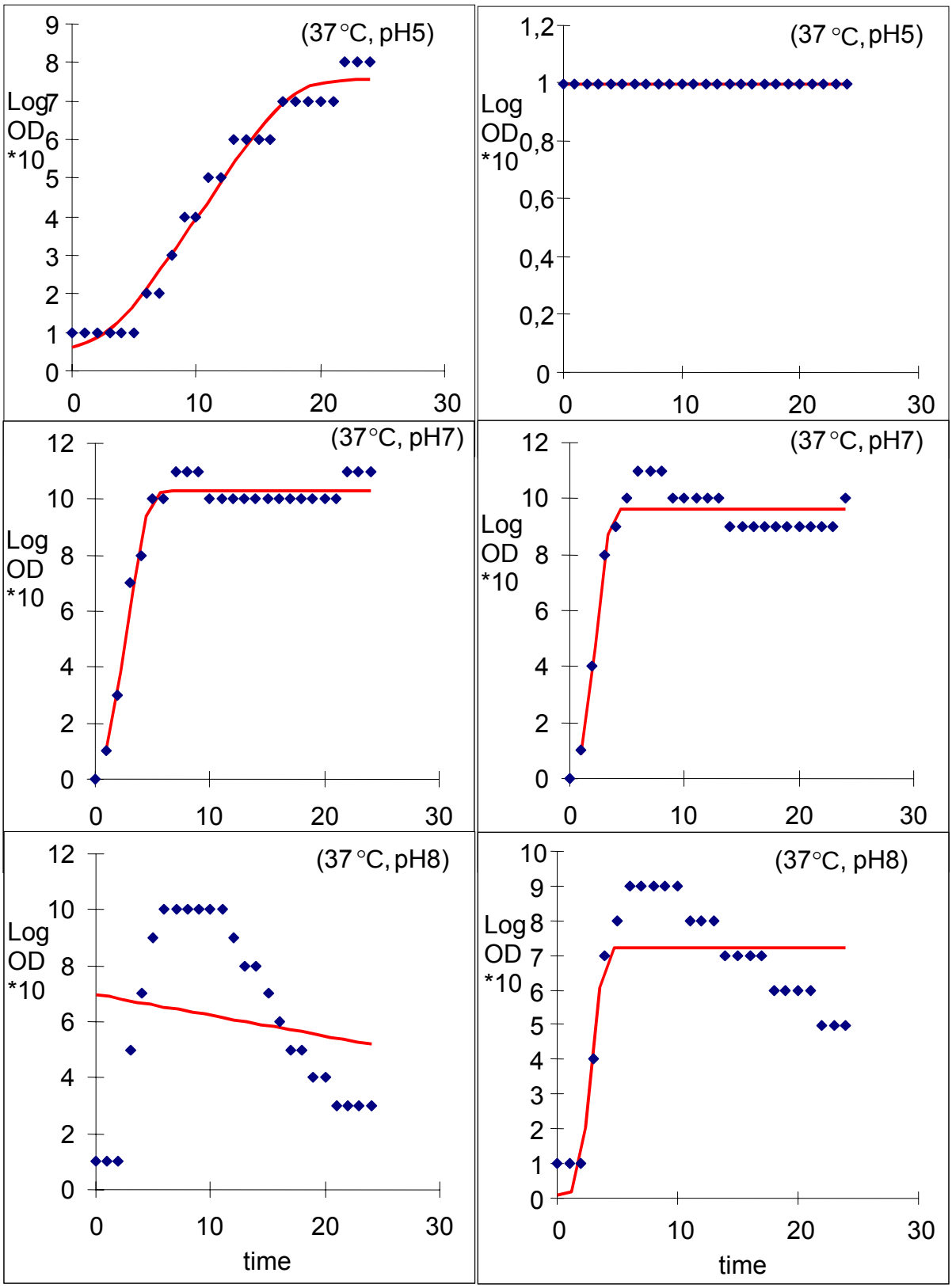


Figure 15 Common (left column) and atypical (right column) growth curves fitted with the Baranyi and Roberts (1994) model to data of B. cereus growth at 37 °C and pH 5 (top row), pH 7 (middle row) and pH 8 (bottom row).

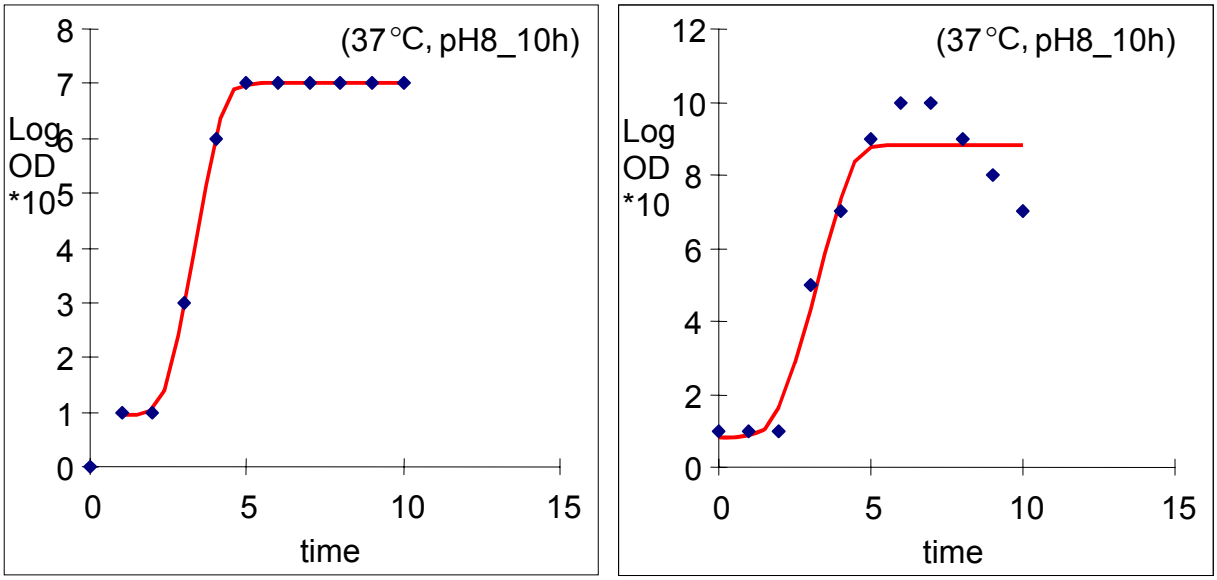


Figure 16 Common (left) and atypical (right) growth curves fitted with the Baranyi and Roberts (1994) model to data of B. cereus growth at 37 °C and pH 8 using only the first 10 hours of the growth data.

Measurements at 24 °C resulted in good fits, although sometimes an OD_{max} was not reached within 24 hours (Figure 14 top right) resulting in no estimate for this parameter. Occasionally a culture grew further after a preliminary constant OD value over a short time span (Figure 14 middle right). And, if the OD value decreased over a short time span after the maximum was reached, the model estimated an OD_{max} somewhat lower than the maximum OD value reached by the culture (Figure 14 bottom right).

At 37 °C and pH 5 some cultures would not start growing at all within 24 hours, resulting in a minimum OD of its initial value, no estimate for the OD_{max} and the lag time, and a growth rate of 0 (Figure 15 top right). At this higher temperature and pH 7, OD_{max} values generally already occurred within the first 10 hours of the experiment (Figure 15 middle). However, as OD values did not drop as much as they did at pH 8, good fits were found in general.

Note that although Figures 14 to 16 show general (left hand side) and particularly found fits (right hand side) for the different experimental set-ups, this does not mean that these results only apply to those experiments. That is, a result as shown in Figure 14 (top) could also have appeared at any other temperature/pH combination, however not as frequently as it was found at 24 °C and pH 5. This also applies to the other results as shown in these figures.

3.5.1.2 Parameter estimates

Table 27 shows the number of growth experiments, at the different temperatures and pH values according to origin of strain, included in the parameter estimates. As each experiment results in a data set independent of others, total numbers as presented in Table 27 were used in the parameter estimations.
Only the biologically relevant parameters, growth rate (OD h^{-1}) and lag time (h), are used in further analysis.

Table 27 Number of growth experiments according to B. cereus strain category and temperature/pH combination.

	Temp: 24 °C, pH: 5, 7, 8 Temp: 37 °C, pH: 5, 7	Total	Temp: 24 °C, pH: 8	Total
Environment	3 * 15	45	4 * 15	60
Food	3 * 35	105	4 * 35	140
Diarrhoeal	3 * 40	120	4 * 40	160
Emetic	3 * 10	30	4 * 10	40
Total	3 * 100	300	4 * 100	400

Figures 17 to 22 show frequency distributions of parameter estimates for strains tested at 24 °C and 37 °C with the different pH values. As can be seen from these figures, the number of parameter estimates is not always equal to the total number of tested strains per group (compare Figures 17 to 22 with Table 27). Occasionally the model was not able to fit the data due to an "ill conditioned problem", *i.e.* the data was too inconsistent to get parameter estimates. Most of the times, however, a value was not assigned to a parameter because the data did not show the particular statistic within 24 hours. If, for example, a food strain did not show any growth, a growth rate of 0 and, subsequently, no lag time was estimated. See Figure 15 (top, right) for an example of such a fit. This resulted in growth rates about 0 and a reduced number of estimated lag times for this category in Figure 17. In other cases, instant growth occurred, which should result in a lag time estimate of 0. Instead this resulted in a lack of estimated lag times. In those cases, however, lag times were assigned a value 0 artificially.

Figure 17 shows that parameter estimates for experiments done at 24 °C and pH 5 are generally independent of strain origin. Lag times show high variability within origin of strains. However, it seems like emetic strains do not have lag times below 8 hours. That is, relatively high lag times causes the maximum OD value not being reached within 24 hours. Accordingly, a relatively frequent number of model fits look like Figure 14 (top right) for these particular experiments. Variability in estimates for the growth rate is low. Yet, a cluster of several strains occurs having growth rate estimates at about 3.5 OD h^{-1} (Figure 17). These

strains are generally associated with relatively high lag times (over 14 hours) followed by instant growth. Most of the times, however, this combined estimate of a high lag time and growth rate only occurs for one out of the three replicates for a particular strain and so these findings cannot be associated with a particular strain in general. Still, one food strain (WSBC 10746, Appendix 1) showed these high parameter estimates when the Baranyi and Roberts (1994) model was fitted to the data of all three experiments. In addition, two environmental strains (WSBC 10538 and 10854, Appendix 1) and one emetic strain (WSBC 10530, Appendix 1) showed relatively high growth parameter estimates for two out of three replicates.

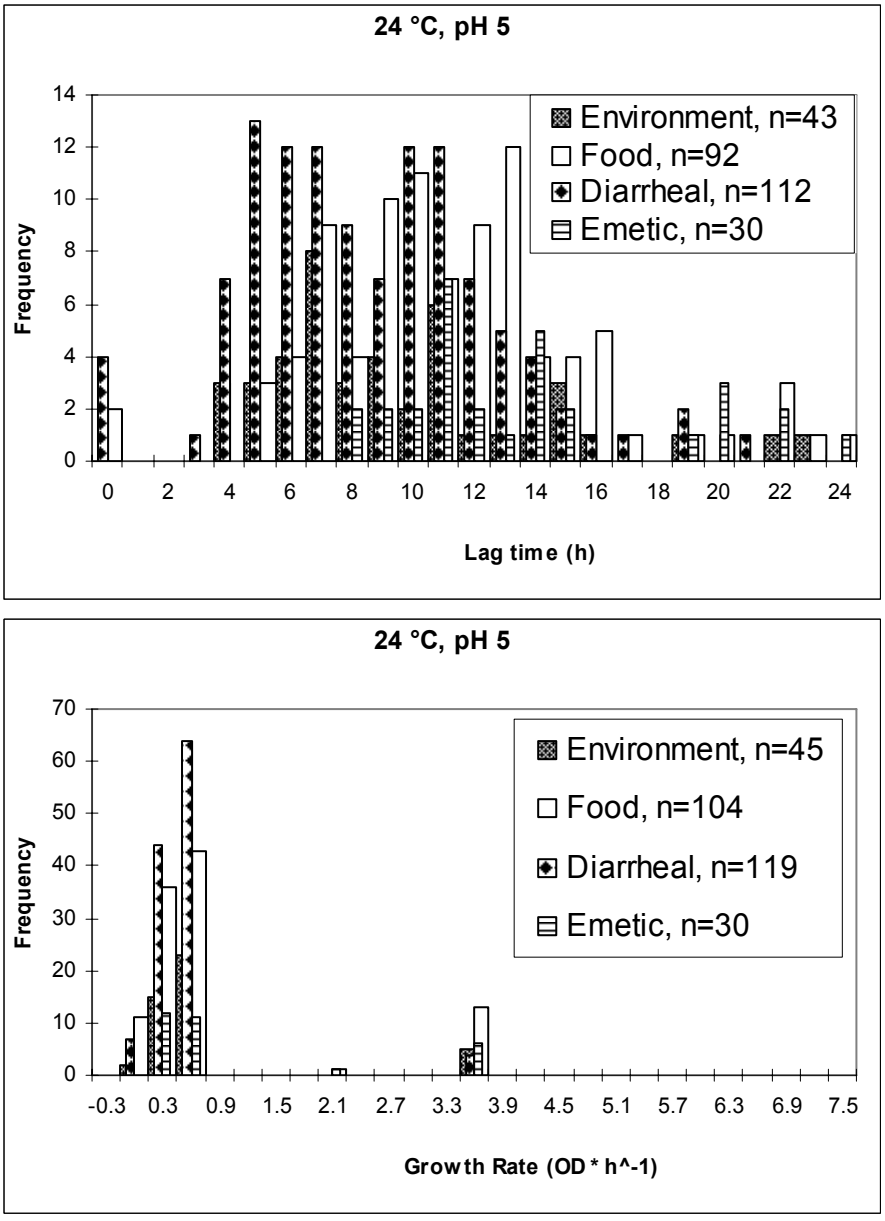
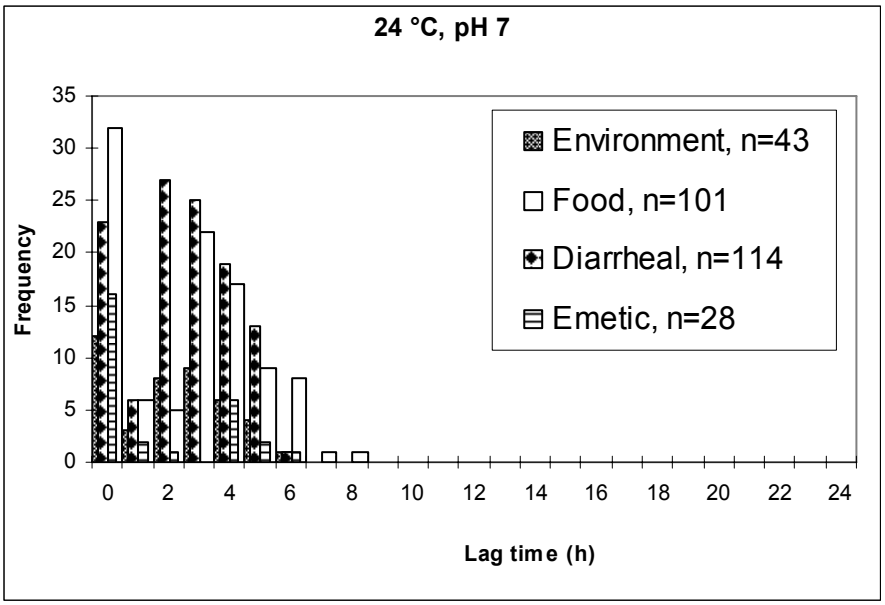


Figure 17 Distribution of estimated lag-times and growth rates, following the Baranyi and Roberts (1994) model, of B. cereus strains growing at 24 °C and pH 5 according to strain category.

Experiments at 24 °C and pH 7 resulted in parameter estimates as shown in Figure 18. There were no clear trends in parameter estimates between strains from different origins. This temperature/pH combination, however, did result in instant growth for many of the tested strains. Emetic strains in particular did not show a clear lag phase. This resulted in assigning a lag time of 0 h. for 16 out of the 30 tested strains. A representative fit for this phenomenon is shown in Figure 14 (middle, right).



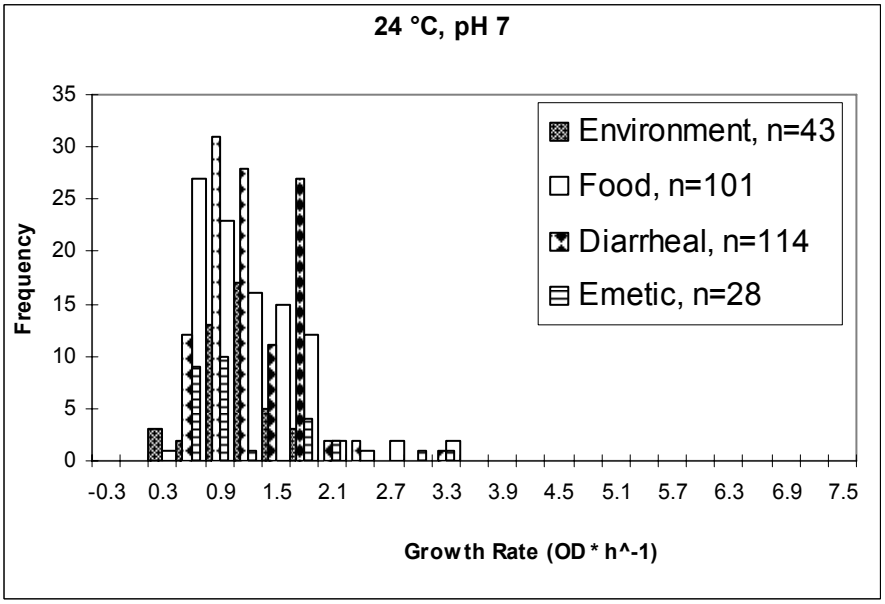
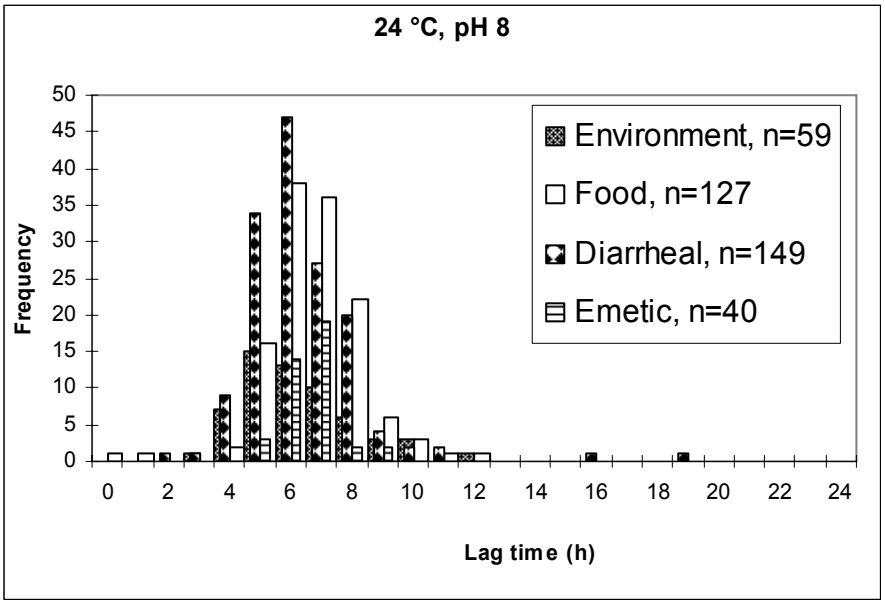


Figure 18 Distribution of estimated lag-times and growth rates, following the Baranyi and Roberts (1994) model, of B. cereus strains growing at 24 °C and pH 7 according to strain category

As already shown in Figure 14 (bottom), the sigmoid growth curve as proposed by Baranyi and Roberts (1994) represented the data from the experiments at 24 °C and pH 8 very well. This resulted in parameter estimates for almost all tested strains. Also, in this case, there does not seem to be a clear difference in parameter estimates for strains from different sources (Figure 19). Occasional large parameter estimates cannot be associated with a particular strain in general in this case as only one of the three replicates showed these parameter estimates for each strain.



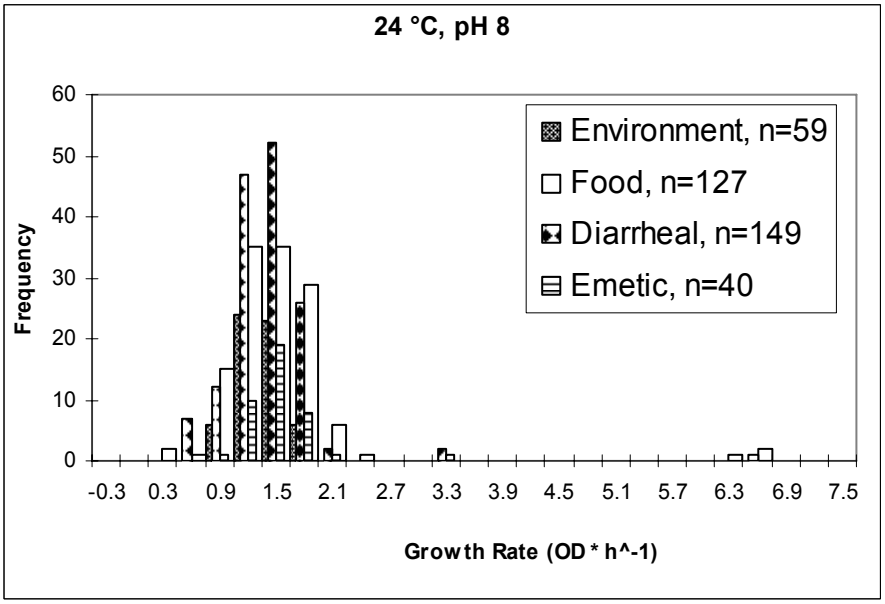


Figure 19 Distribution of estimated lag-times and growth rates, following the Baranyi and Roberts (1994) model, of B. cereus strains growing at 24 °C and pH 8 according to strain category

Figure 20 shows the parameter estimates of data from experiments at 37 °C and pH 5. This Figure shows a huge variability in estimated lag times. Growth rates are centered about 0 and estimated growth rates for emetic strains are, with the exception of 2 strains, all 0. Figure 15 (top, right) represents a fit to this particular type of data. Subsequently, a lag time could often not be estimated, particularly for the emetic strains. The two emetic strains, for which a lag time of respectively 13 and 23 hours was estimated, correspond to the two strains having a nonzero growth rate of 3.5 and 1.9 OD hours^{-1}respectively. These particular results are due to bacteria of these strains showing no growth for a long period and suddenly, after 13 and 23 hours, some growth occurs. One of the food strains (WSBC 10466, Appendix 1) appeared to have relatively high growth rates (and lag times of about 6.5 hours) for all three replicates.

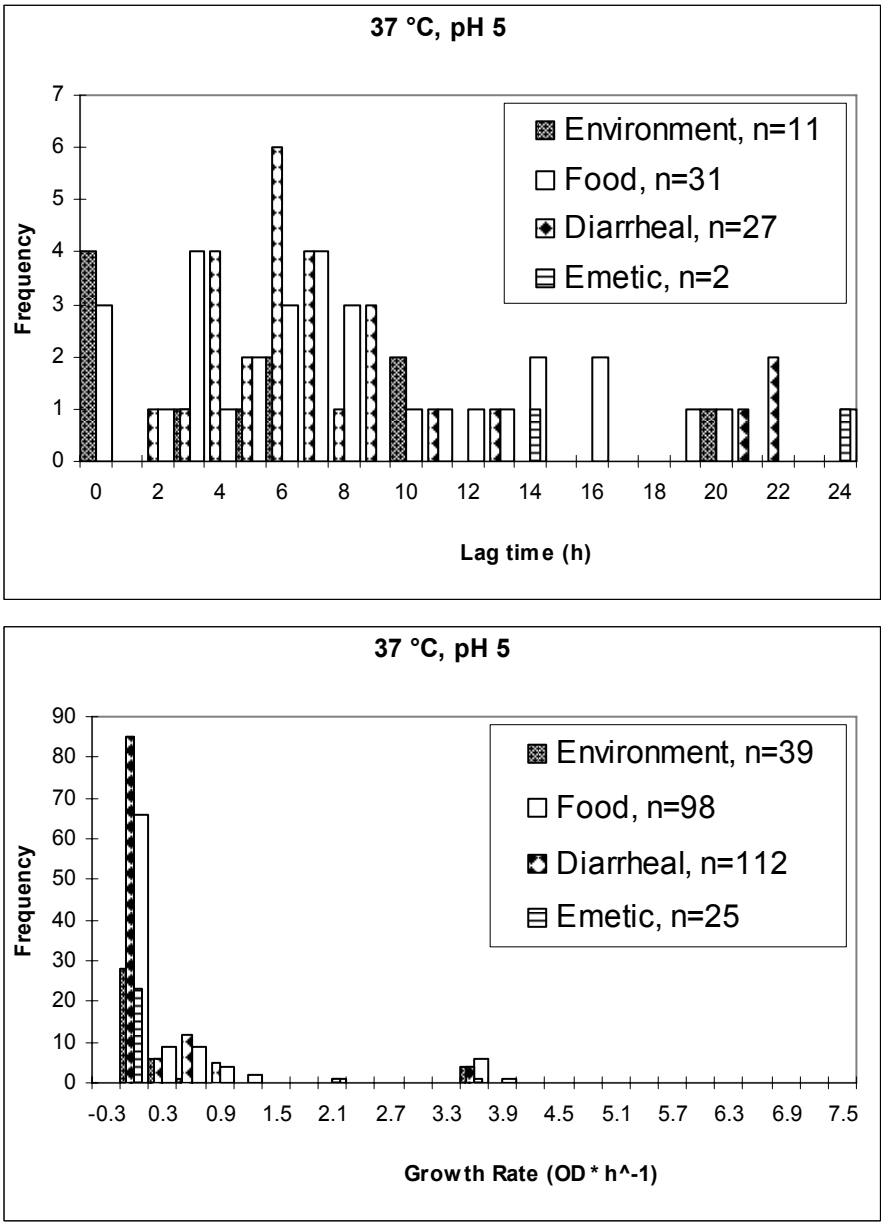


Figure 20 Distribution of estimated lag-times and growth rates, following the Baranyi and Roberts (1994) model, of B. cereus strains growing at 37 °C and pH 5 according to strain category

As the model fitted the data reasonably well for the 37 °C and pH 7 experiments almost all strains have parameter estimates. Figure 15 (middle) shows that bacteria start growing almost instantly after the start of the experiment, causing lag times to be either very low or not estimated and so 0 hours was often assigned (Figure 21).

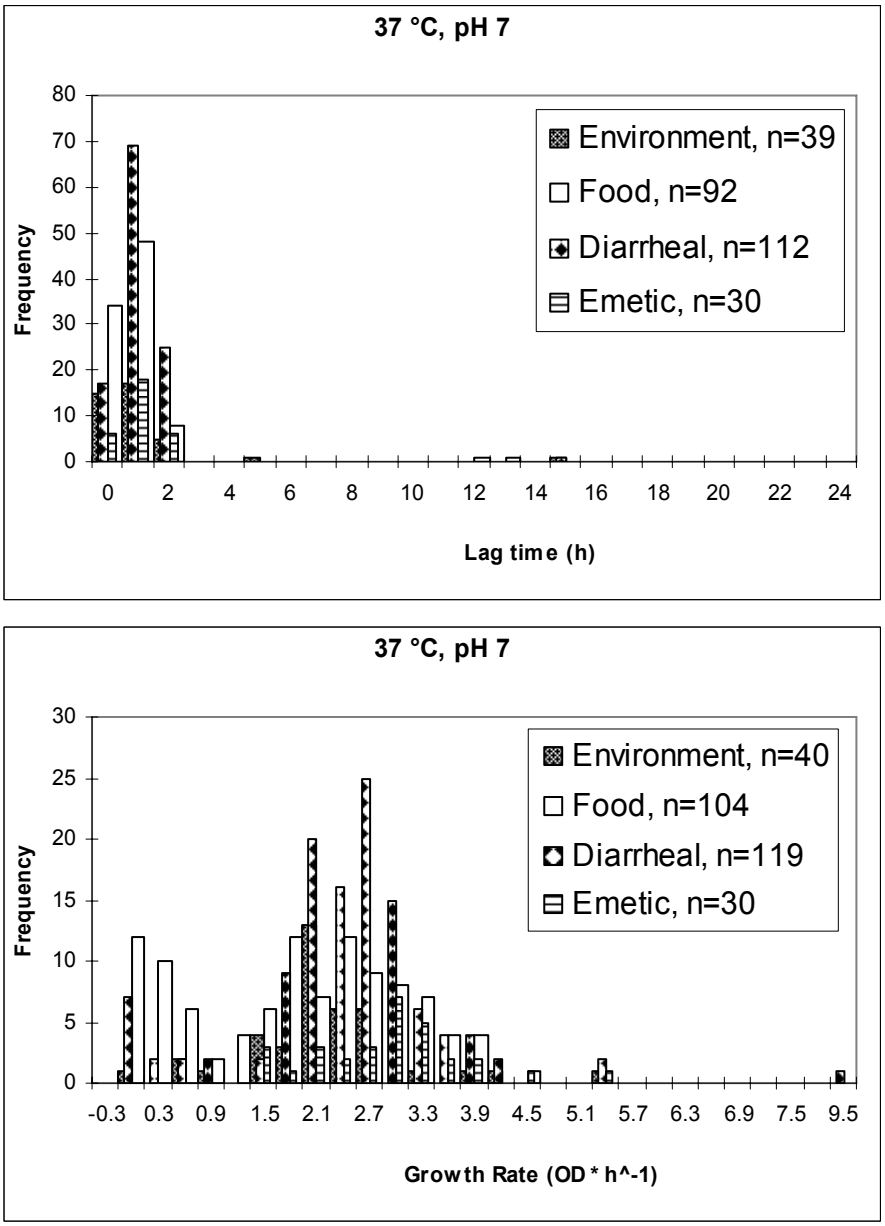


Figure 21 Distribution of estimated lag-times and growth rates, following the Baranyi and Roberts (1994) model, of B. cereus strains growing at 37 °C and pH 7 according to strain category

Figure 15 (bottom, left) already shows the typical model fit with a negative slope for cultures grown at 37 °C and pH 8. This result can also be seen from the parameter estimates in

Figure 22. The model estimates either negative growth rates or relatively high growth rates resulting in a reduced number of lag time estimates. This result indicates the model does not fit the data very well. OD_{max} values are generally reached within 10 hours (Figure 15, bottom), therefore, parameter estimates were repeated with data up to 10 hours which releases the weight on low OD values after the maximum has been reached.

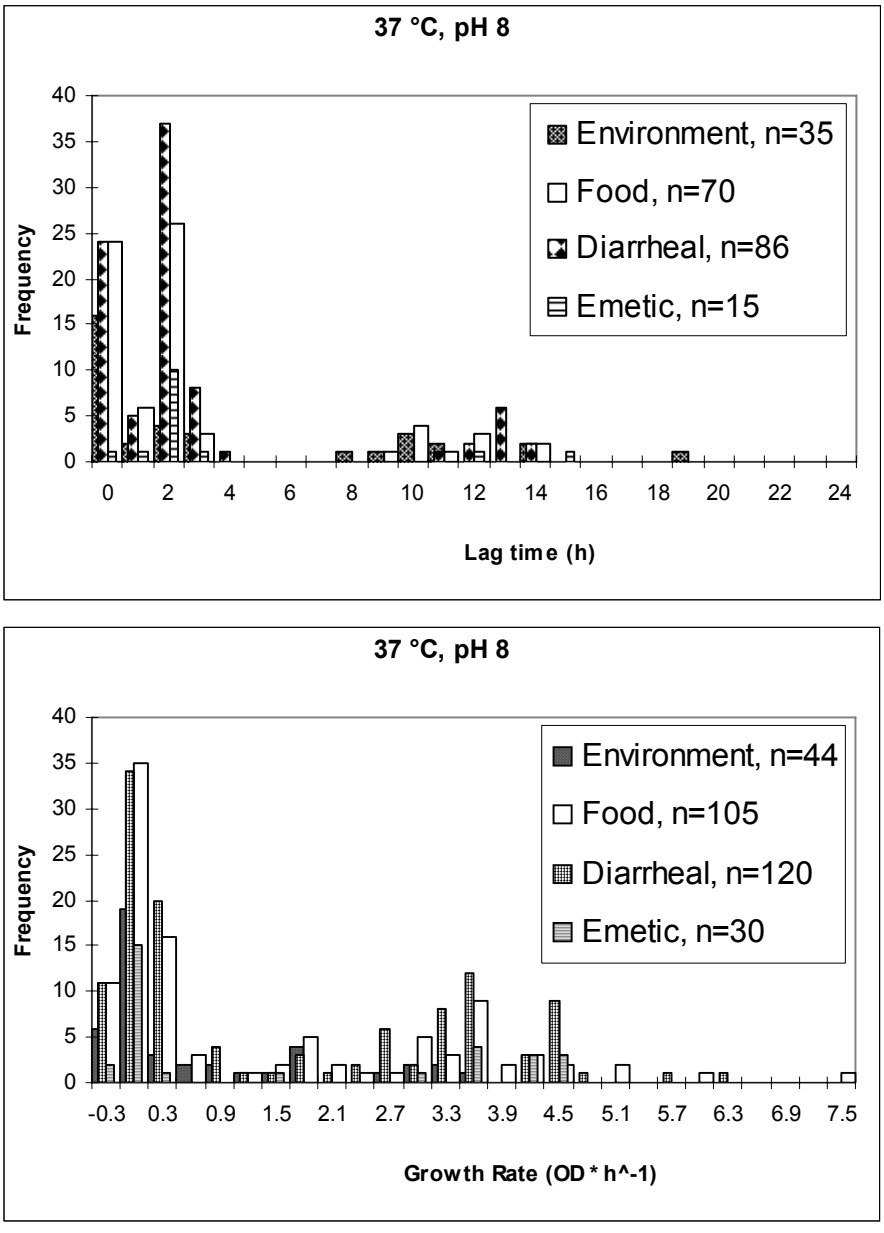


Figure 22 Distribution of estimated lag-times and growth rates, following the Baranyi and Roberts (1994) model, of B. cereus strains growing at 37 °C and pH 8 according to strain category

Figure 23 shows the new parameter estimates when the Baranyi and Roberts (1994) model was fitted to data containing measurements up to 10 hours at 37 °C and pH 8. Negative growth rates have disappeared, and parameter estimates were found for almost all strains.

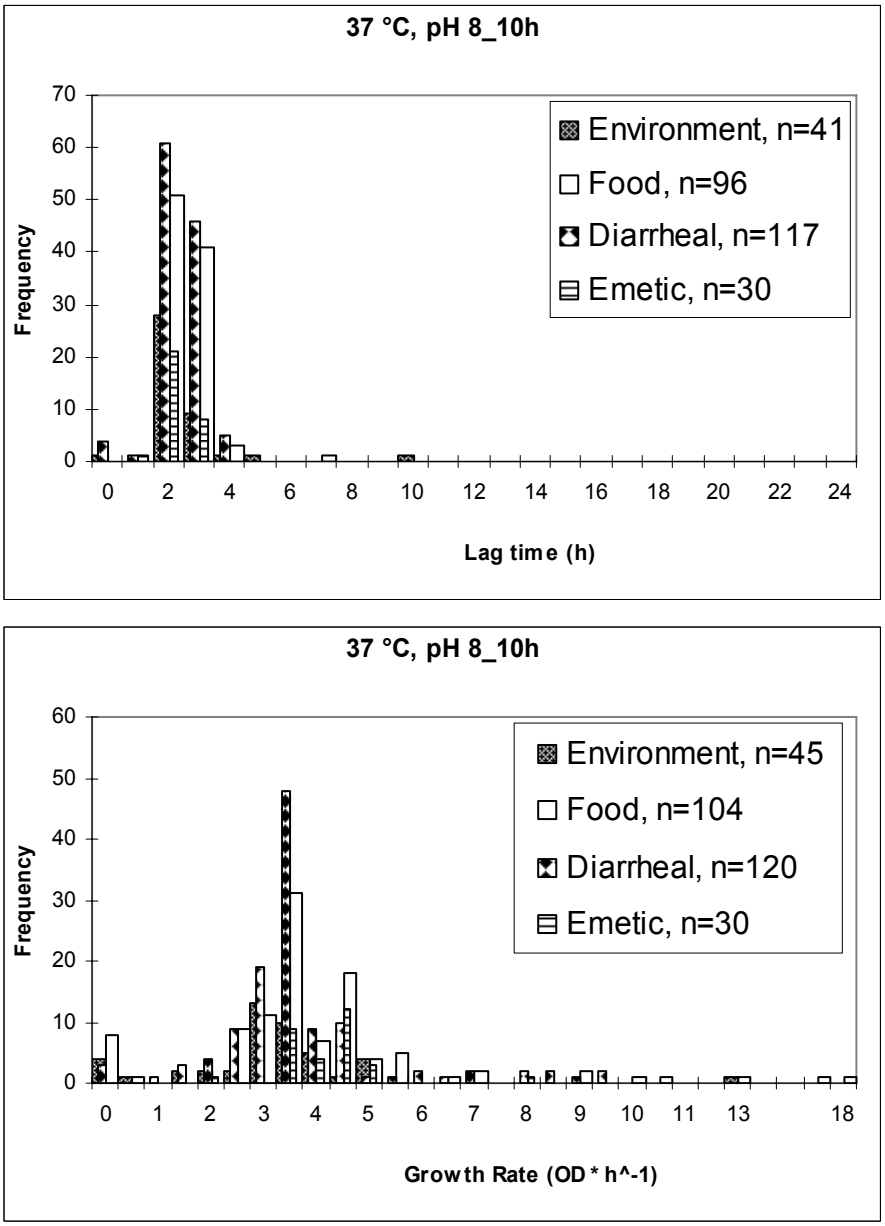


Figure 23 Distribution of estimated lag-times and growth rates, following the Baranyi and Roberts (1994) model, of B. cereus strains growing at 24 °C and pH 8 according to strain category (when only the first 10 hours of the growth data is used).

Figures 17 to 23 show, in general, no clear visible difference between environmental, food, diarrhoeal and emetic strains within a certain temperature/pH combination. Yet,

- emetic strains show relatively large lag times at 24 °C/pH 5,

- a majority of the emetic strains show instant growth at 24 °C/pH 7,
- all strains, but emetic strains in particular, show generally no growth at 37 °C/pH 5, and
- many strains show instant growth at pH 7 in combination with both temperatures. Hence, neutral pH seems to be related to an enhanced probability of growth.

3.5.2 Data analysis

Figures 17 to 23 show the results on the parameter estimates lag-time and growth rate for *B. cereus*. Average parameter values were calculated per strain category at the two temperatures, 24 and 37 °C and three pH values, pH 5, 7 and 8. Analysis of Variance (ANOVA) tests reveal differences between strain categories that are not immediately evident from Figures 17 to 23.

Differences between groups at 24 °C and pH 5

Anova tests show that both average lag times and growth rates are significantly ($\alpha = 0.05$) different between groups. Diarrhoeal strains show to have the lowest growth rate and tested emetic strains have an, on average, highest growth rate (Table 28).
Figure 17 already showed that all tested emetic strains have lag-times above 8 hours. A test shows compelling evidence that average lag-times differ between categories of tested strains (Table 29). Furthermore, Table 29 shows the relatively high lag-times for the emetic and low lag-times for the diarrhoeal strains.

Table 28 Results of an Analysis of Variances (ANOVA) test to reveal differences in estimated growth rate of B. cereus strains (n) at 24 °C and pH 5.

Growth rate per category	n	average	variance (s^2)	F	P-value
Environment	45	0.64	1.04	3.61	0.014
Food	104	0.68	1.17		
Diarrhoeal	119	0.40	0.35		
Emetic	30	0.97	1.72		

Table 29 Results of an Analysis of Variances (ANOVA) test to reveal differences in lag time of B. cereus strains at 24 °C and pH 5.

Lag-time per category	n	average	variance (s^2)	F	P-value
Environment	43	9.19	20.3	14.7	< 0.001
Food	92	10.78	19.4		
Diarrhoeal	112	8.12	15.1		
Emetic	30	13.31	19.8		

Differences between groups at 24 °C and pH 7

Average growth rates and lag-times of *B. cereus* do not differ significantly between environmental, food, diarrhoeal and emetic strains as tested at 24 °C and pH 7 (Tables 30 and 31).

Table 30 Results of an Analysis of Variances (ANOVA) test to reveal differences in estimated growth rate of B. cereus strains (n) at 24 °C and pH 7.

Growth rate per category	n	average	variance (s^2)	F	P-value
Environment	43	0.94	0.11	1.67	0.174
Food	101	1.02	0.34		
Diarrhoeal	114	1.13	0.22		
Emetic	28	1.04	0.54		

Table 31 Results of an Analysis of Variances (ANOVA) test to reveal differences in estimated lag-time of B. cereus strains (n) at 24 °C and pH 7.

Lag-time per category	n	average	variance (s^2)	F	P-value
Environment	43	1.83	2.62	1.93	0.125
Food	101	2.20	3.82		
Diarrhoeal	114	2.07	2.26		
Emetic	28	1.35	3.71		

Differences between groups at 24 °C and pH 8

Both growth rates and lag-times do not differ significantly between groups of *B. cereus* strains tested at 24 °C and pH 8 (Tables 32 and 33).

Table 32 Results of an Analysis of Variances (ANOVA) test to reveal differences in estimated growth rate of B. cereus strains (n) at 24 °C and pH 8.

Growth rate per category	n	average	variance (s^2)	F	P-value
Environment	59	1.21	0.054	1.68	0.170
Food	127	1.40	0.732		
Diarrhoeal	149	1.28	0.336		
Emetic	40	1.39	0.133		

Table 33 Results of an Analysis of Variances (ANOVA) test to reveal differences in estimated lag time of B. cereus strains (n) at 24 °C and pH 8.

Lag-time per category	n	average	variance (s^2)	F	P-value
Environment	59	5.74	3.15	1.31	0.271
Food	127	6.21	2.56		
Diarrhoeal	149	5.92	3.69		
Emetic	40	6.14	0.75		

Differences between groups at 37 °C and pH 5

Tables 34 and 35 show that only average lag-times differ significantly between tested groups of *B. cereus* strains. There is compelling evidence that emetic strains have the highest lag times. Environmental strains have the lowest lag-times (Table 35). However, as only 2 lag times were estimated for the emetic strains (see Section 3.5.1.2 for explanation) this analysis is not valid. An Anova test comparing average lag-times of environmental, food and diarrhoeal strains was performed instead. The F-value and P-value for this test are shown in Table 39 between brackets. A P-value of 0.007 shows a significant difference between the average lag-times for the 3 categories. That is, food strains have the highest (7.83 hours) and environmental strains have the lowest lag time (2.94 hours).

Table 34 Results of an Analysis of Variances (ANOVA) test to reveal differences in estimated growth rate of B. cereus strains (n) at 37 °C and pH 5.

Growth rate per category	n	average	variance (s^2)	F	P-value
Environment	39	0.39	1.12	0.819	0.484
Food	98	0.36	0.84		
Diarrhoeal	112	0.21	0.43		
Emetic	25	0.21	0.60		

Table 35 Results of an Analysis of Variances (ANOVA) test to reveal differences in estimated lag time of B. cereus strains (n) at 37 °C and pH 5.

Lag-time per category	n	average	variance (s^2)	F	P-value
Environment	19	2.94	25.78	6.46	< 0.001
Food	32	7.83	35.56	(5.35)	(0.007)
Diarrhoeal	27	7.65	29.30		
Emetic	2	18.42	50.93		

Differences between groups at 37 °C and pH 7

Only calculated average growth rates differ significantly between groups of the tested *B. cereus* strains at 37 °C and pH 7 (Tables 36 and 37). The food group shows the, on average, lowest growth rate and strains that potentially cause emesis have the highest growth rates. The compelling evidence for differences in growth rates (P<0.001, Table 36) is probably due to the small variances.

Table 36 Results of an Analysis of Variances (ANOVA) test to reveal differences in estimated growth rate of B. cereus strains (n) at 37 °C and pH 7.

Growth rate per category	n	average	variance (s^2)	F	P-value
Environment	40	2.05	0.88	10.56	<0.001
Food	101	1.78	1.33		
Diarrhoeal	114	2.44	1.18		
Emetic	30	2.80	0.81		

Table 37 Results of an Analysis of Variances (ANOVA) test to reveal differences in estimated lag time of B. cereus strains (n) at 37 °C and pH 7.

Lag-time per category	n	average	variance (s^2)	F	P-value
Environment	39	0.87	5.65	0.23	0.875
Food	92	0.65	3.07		
Diarrhoeal	112	0.72	1.97		
Emetic	30	0.58	0.26		

Differences between groups at 37 °C and pH 8_10h

Anova tests show no significant differences (α=0.05) between groups for the different growth characteristics as estimated with the Baranyi and Roberts (1994) model for the selected *B. cereus* strains (Tables 38 and 39).

Table 38 Results of an Analysis of Variances (ANOVA) test to reveal differences in estimated growth rate of B. cereus strains (n) at 37 °C and pH 8, using data of the first 10 hours in the growth experiment.

Growth rate per category	n	average	variance (s^2)	F	P-value
Environment	45	2.97	3.54	2.56	0.055
Food	104	3.89	7.29		
Diarrhoeal	120	3.48	2.65		
Emetic	30	3.96	0.88		

Table 39 Results of an Analysis of Variances (ANOVA) test to reveal differences in estimated lag time of B. cereus strains (n) at 37 °C and pH 8, using data of the first 10 hours in the growth experiment.

Lag-time per category	n	average	variance (s^2)	F	P-value
Environment	41	2.08	1.89	1.19	0.313
Food	96	2.11	0.38		
Diarrhoeal	117	1.95	0.36		
Emetic	30	1.89	0.23		

Except for experiments at 24 °C/pH 5 and 37 °C/pH 7, average growth rates do not differ significantly between tested groups of *B. cereus* strains at the different temperature/pH combinations. Average lag times show a significant difference between groups in 2 of the 6 experiments, that is, at 24 °C/pH 5 and at 37 °C/pH 5 (Tables 28 to 39). Table 40 gives a summary of Tables 28 to 39 pointing out those categories with an, on average, lowest/highest growth rate and lag time for those test results that showed significant differences. Although diarrhoeal strains show both low growth rates and lag times at 24 °C/pH5, correlation is weak (r=-0.06) for these growth characteristics of diarrhoeal strains. Analogously, high growth rates do, (with r=0.44) not seem to correspond to high lag times for emetic strains at 24 °C/pH 5.

Table 40 Summary of Tables 28 to 39 showing significant and not significant differences (n.s.d. with accompanying P-value) in estimated growth rates and lag times for B. cereus strains growing at 24°C/37°C and pH5/pH7/pH8. The < and > signs show the strain categories having the lowest and highest parameter estimates respectively per temperature/pH combination.

24 °C	pH 5		pH 7	pH 8
	<	>		
Growth rate	diarrhoeal	emetic	n.s.d. P=0.174	n.s.d. P=0.170
Lag-time	diarrhoeal	emetic	n.s.d. P=0.125	n.s.d. P=0.271

37 °C	pH 5		pH 7		pH 8
	<	>	<	>	
Growth rate	n.s.d. P=0.484		food	emetic	n.s.d. P=0.055
Lag-time	environ-ment	emetic (n=2) (food)	n.s.d P=0.875		n.s.d. P=0.013

3.6 *B. cereus* and food source

In addition to the four strain categories environment, food, diarrhoeal and emetic, a more detailed description was given on the origin of the 100 selected *B. cereus* strains (Appendix 1). For example, product information was collected for the 85 *B cereus* strains selected from food sources. Figure 24 gives an overview of the contribution of generalized food products that led to cases of diarrhea and/or emesis. An overview of the possible food poisoning product association with diarrhoeal and/or emetic strains from this limited strain selection is given in the legend of Figure 24. It is shown, for example, that rice dishes led more times to diarrhoea than to emesis. On the other hand, milk only resulted in cases of emesis.This first screening gives indications for more in depth research on topics like

- Are vegetables generally not associated with emesis from *B. cereus*?
- Do rice dishes with *B.cereus* give more rise to diarrhoea than to emesis?
- Are baby foods associated with emetic strains?
- etc.

Note, however, that the number of diarrhoeal strains selected for this study is 4 times higher than the number of strains that have shown to have caused emesis (40 and 10, respectively).

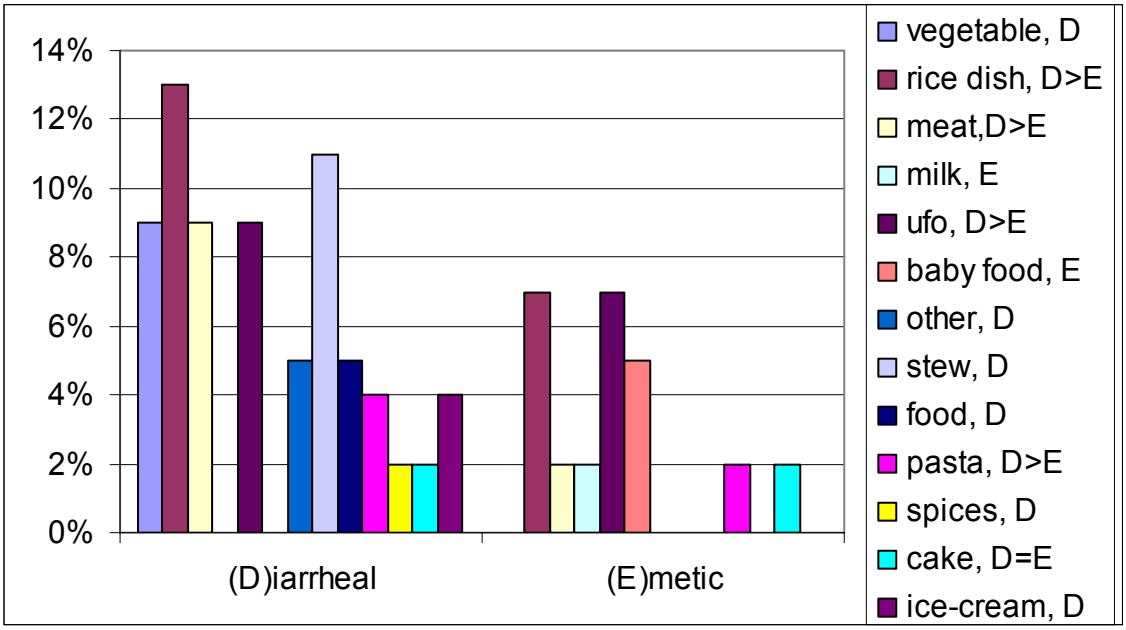


Figure 24 Relative abundance (%) of diarrhoeal (D) and emetic (E) strains according to the products that led to food poisoning (ufo= unidentified food object).The capitals D and E in the legend refer to the relative abundance of D compared to E strains in the different food sources.

3.7 Correlation between physical features of *B. cereus* strains

The potential hazard of strains can be identified by correlating minimum growth temperatures (*i.e.* 4, 7, 10, ≥ 10 °C) to several other relevant biophysical characteristics. For example, a positive correlation between minimum growth temperature and maximum growth temperature would indicate one could assign strains to growth temperature groups (*e.g.* mesophilic and psychrotolerant strains). A positive correlation between minimum growth temperatures and D-values would indicate that strains which are able to grow at 4 °C have small D-values, *i.e.* are rapidly inactivated during cooking and so may be less harmful for public health. Relating minimum growth temperatures to enterotoxin production could reveal information on whether strains with a minimum growth temperature at, *e.g.* 10 °C can produce relatively more enterotoxins. This would indicate that keeping these particular food products at fridge temperatures would be less harmful.
Table 41 gives an overview of Spearman's Rank Correlations between minimum growth temperature and other characteristics for the whole strain set and the subsets environment, food, emetic and diarrhoeal strains. Rank correlations are given to reveal a relationship that can be linear or non-linear, to diminish the effect of extreme values and to be able to include growth temperatures with "≥/≤"-values, which are then given a rank.
Correlations between –0.5 and 0.5 indicate "weak" correlation, between ± 0.5 and ±0.8 indicates moderate correlation.
Table 41 shows there is only few moderate correlations (underlined). These moderate correlations are visualised in Figures 25 to 27.

Table 41 Rank correlations between minimum growth temperature and maximum growth temperature, D-value and enterotoxin production for the100 B. cereus strain set and subgroups.

	100 set	Environ.	Food	Emetic (17)	Emetic (10)	Diarrhoea
Max. growth temp.	0.44	0.65	0.39	0	0	0.23
D-value	0.44	0.18	0.56	0.07	0.57	0.20
Diarrhoeal	0.09	0.12	0.16	-0.10	-0.03	-0.06
Titer HBL-L2	-0.01	-0.71	0.08	-[1]	-	-0.25
Antigen titer HBL-B	-0.06	-0.61	0.09	-	-	-0.26
Antigen titer NHE-B	0.15	0.20	0.08	0.01	0.34	0.03

1 '-' not applicable

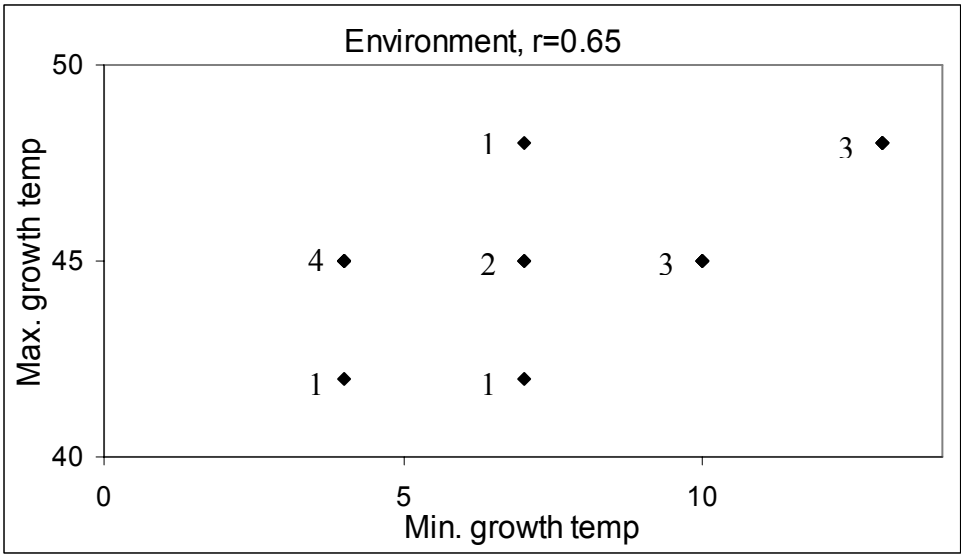


Figure 25 Relationship between minimum growth temperature and maximum growth temperature for environmental B. cereus strains. Numbers indicate the number of overlapping strains at each point.

A fair amount of the environmental strains can be further classified according to growth temperatures (Figure 25). The combined property of emetic strains having both large *D*-values (Table 17) and relatively high minimum growth temperatures (Table 3) results in a moderate correlation between these features (Figure 26). This implies that emetic strains in food products which are kept outside the fridge can replicate. On top of that, those strains that are able to grow in these conditions are also slowly heat inactivated and thus potentially very hazardous. That is, production of emetic toxin by higher numbers of cells during a longer time period. A similar correlation was found for the food strains (Figure 26). Finally, environmental strains show a moderate correlation between minimum growth temperature and HBL-L2/B production (Figure 27).

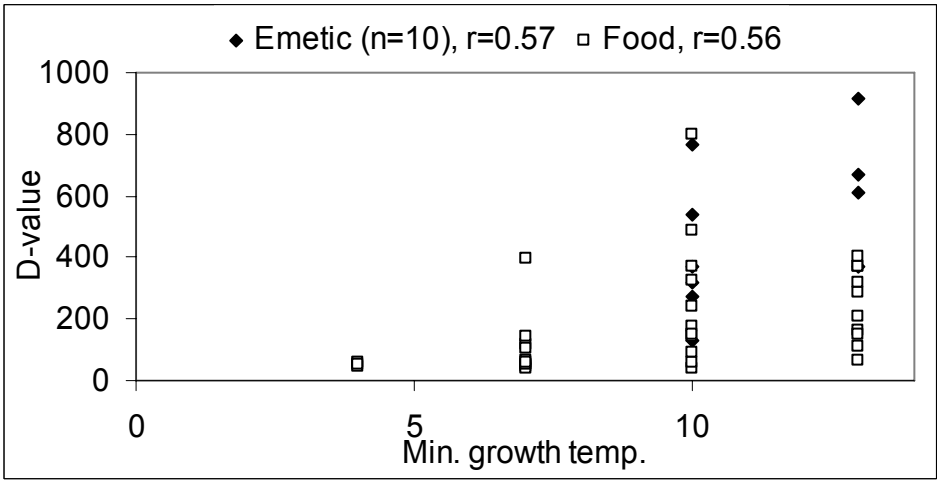


Figure 26 Relationship between minimum growth temperature and estimated decimal reduction time (D-value) following eq.1 for the 10 B. cereus strains selected from emetic food poisoning sources and the food strains.

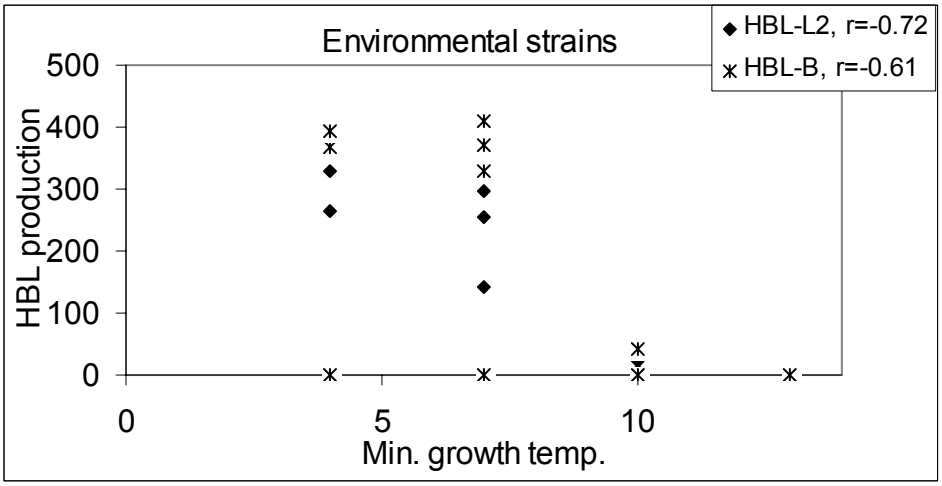


Figure 27 Relationship between minimum growth temperature and HBL-L2/B production for environmental B. cereus strains.

Table 42 shows the correlation coefficients between the two parameters, *d* and *p*, of the non-linear inactivation model (eq.2) for *B. cereus* strains. Moderate correlations are shown in Figures 28 and 29. Figure 28 shows that environmental strains can actually very well be described by a non-linear inactivation model. Concave curves ($p < 1$) agree with a short time until first decimal reduction (small *d*) and *visa versa*. If the two relatively large *d*-values were to be omitted, a r-value of 0.92 would be obtained, indicating a strong correlation here.

Although emetic strains seem to show a moderate negative correlation between *d* and *p*, Figure 29 shows this relation to be mainly caused by the two extreme *d*- and *p*-values.

Table 42 Rank correlation between the estimated time until first decimal reduction (d-value) and the shape parameter (p-value) of model eq.2 for the different B. cereus strain categories.

100 strains	0.18
Environmental strains	0.65
Food strains	0.16
Emetic strains (17)	-0.09
Emetic strains (10)	-0.54
Diarrhoeal strains	0.31

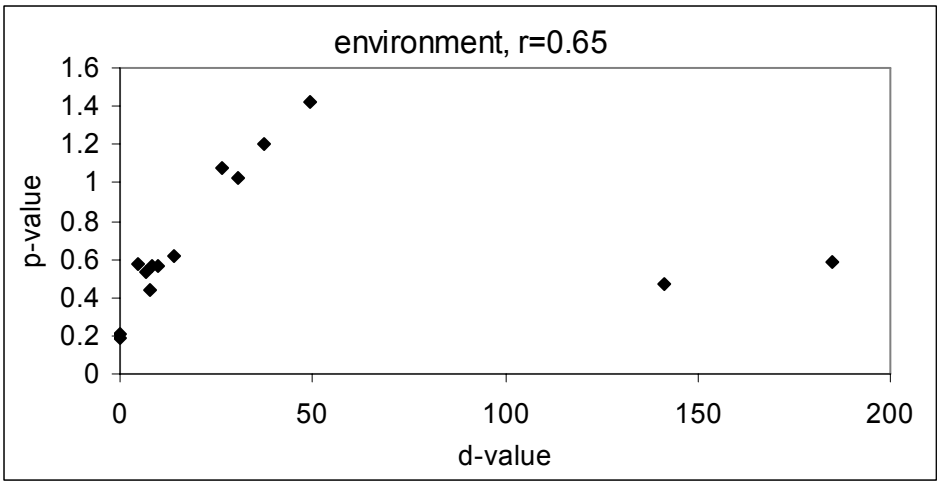


Figure 28 Relationship between the estimated time until first decimal reduction (d-value) and the shape parameter (p-value) of model eq.2 for the environmental B. cereus strains.

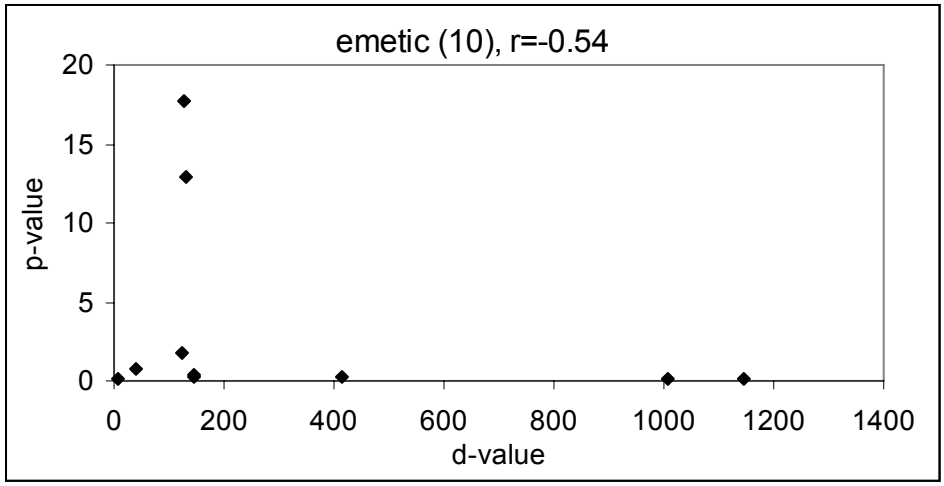


Figure 29 Relationship between the estimated time until first decimal reduction (d-value) and the shape parameter (p-value) of model eq.2 for the 10 emetic B. cereus strains selected from food poisoning cases.

4. General conclusions

Biophysical characterisation of the foodborne bacteria *B. cereus* has been conducted experimentally using a subset of 100 *B. cereus* strains selected from the environment, food and food poisoning (diarrhoeal and emetic strains) categories. Experimental results and additional statistical analysis have lead to the following general conclusions concerning potential hazardous *B. cereus* strains in Europe.

4.1 Growth temperatures

- There is a significant difference between categories in minimum growth temperatures in general. Emetic and diarrhoeal strains have the largest contribution in this difference, *i.e.*:

  - Food poisoning strains do not grow at 4 °C, which is significantly different from the non-food poisoning category having 9/50 strains with a minimum growth temperature at 4 °C.
  - A majority of the diarrhoeal strains (72 %) has a minimum growth temperature at 7 °C, which is significantly different from the other strain categories at this temperature.
  - A 95 % Confidence Interval for the proportion of diarrhoeal strains having a minimum growth temperature at 7 °C is [0.59 – 0.86].
  - Emetic strains have minimum growth temperatures ≥ 10 °C.

- Maximum growth temperatures also differ significantly between strain categories. Now emetic and environmental strains have the largest impact on this difference, *i.e.*:

  - Emetic strains have a maximum growth temperature ≥ 48 °C, which has a significant impact when compared to other strain categories at this temperature.
  - Environmental strains have significantly low maximum growth temperatures (73 % < 48 °C) compared to other categories.

4.2 Toxin production

- Emetic toxin producing strains are found in all of the categories environment, food and food poisoning.

- Almost all strains (> 90 %) produce enterotoxins.

- The cytK gene was found significantly more in diarrhoeal strains (57 %) than in the other categories. Testing subsequent *B. cereus* strains on cytK gene possession can, therefore, be an indicator for potential food poisoning.

- Significantly little emetic strains possess the cytK gene compared to the other categories.

- Although there is no significant difference between strain categories in Hbl-operon possession, yet

  - Emetic strains do not produce HBL-L2, nor HBL-B, causing a significant difference when comparing strain categories for these proteins.
  - Tests for independence revealed that environmental strains produce relatively more HBL-L2 and diarrhoeal strains produce more HBL-B compared to the other categories.

- Almost all strains (99 %) possess ≥ 1 gene of the Nhe operon and, with that, produce NHE-A (95 %) and NHE-B (92 %).

4.3 Heat resistance

- Spores of emetic strains show relatively high decimal reduction times (*D*-values) and so more heat resistant than the other strains. Although more emetic strains need to be tested to show a significant difference, preliminary calculated 95 % C.I.'s show emetic strains to have higher *D*-values than other strain categories.

- Emetic strains also show relatively long times until first decimal reduction (*d*-values). However, in this case, preliminary 95 % C.I.'s do not confirm a difference between strain categories.

- There is only moderate correlation between *D*-values and *d*-values for the emetic strains (r_s=0.63).

- Environmental strains show, on average, relatively low *d*-values. However, this is again not confirmed when comparing preliminary C.I.'s for the different strain categories.

- Over 70 % of the *B. cereus* strains show concave shaped inactivation curves ($p < 1$) independent of strain origin. This involves a risk when heat inactivation would be based on a linear concept for future strain characterisation. Inactivation time is proportional

with time in a linear concept and will, therefore, be reached sooner as would be predicted in a non-linear concept where inactivation is represented by a tailing curve in time. Moreover, a significant number of food strains have a *p*-value < 1.

4.4 Germination

- Although more strains should be tested, still preliminary 95 % C.I.'s show that:

  - Germination of emetic strains is relatively low (72 (58) %) at 7 °C compared to other categories with average germination percentages > 90 %.
  - Germination of diarrhoeal strains is high (> 99 %) at 30 °C compared to other categories at this temperature.

4.5 Growth characteristics

- Lag times are significantly different between strain categories at 24 °C/pH 5. Emetic strains show to have relatively high lag-times ($\bar{x}$ = 13 h), whereas diarrhoeal strains have relatively short lag times ($\bar{x}$ = 8 h) compared to the other categories.

- The previous association also applies to growth rates at 24 °C/pH 5, with relatively high growth rates for the emetic strains and low growth rates for diarrhoeal strains.

- Still, no correlation exists between growth rate and lag-time for diarrhoeal strains (r_s=-0.06) and only a weak correlation for the emetic strains (r_s=0.44) was found at 24 °C/pH 5.

- Most of the strains show instant growth at 24 °C/ 37 °C and pH 7.

- A significant difference between the 3 strain categories (environment, food and diarrhoeal) became apparent for lag-times of the *B. cereus* strains when kept at 37 °C/pH 5. Food strains show to have relatively high lag times ($\bar{x}$ = 8 h); environmental strains have relatively short lag-times (3 h). (emetic strains were omitted in the test procedure as only 2 strains resulted in estimated lag times)

- Finally significant differences were found between strain categories concerning growth rates at 37 °C/pH 7, where food strains showed to have low average growth rates and emetic strains to have relatively high growth rates.

4.6 Correlation between physical features of *B. cereus* strains

- Only environmental strains show moderate correlation between minimum growth temperatures and:

  - Maximum growth temperature (r_s=0.6), indicating an equal representation of psychrotolerant and mesophilic strains in this category.
  - HBL-L2 production (r_s=-0.7), which can give rise to potentially hazardous situations when strains with relatively low minimum growth temperatures are being stored in the fridge. These strains will not only multiply fast at these temperatures but, with that, produce relatively high amounts of HBL-L2.
  - HBL-B production (r_s=-0.6), having the same implications as for the HBL-L2 enterotoxin.

- Only the 10 emetic strains, selected from food poisoning cases, show a moderate correlation between minimum growth temperatures and:

  - Decimal reduction times (*D*-values, r_s=0.6), indicating an increased hazard for emetic strains having relatively high minimum growth temperatures that are being cooked for a short time.

- Finally, a moderate correlation between time until first decimal reduction (*d*-value) and the shape parameter (*p*-value) of the estimated inactivation curve has been demonstrated for:

  - environmental strains (r_s=0.6) This shows that environmental strains can best be described by a non-linear shaped inactivation curve.
  - Emetic strains (r_s=-0.5).

5. Recommendations

Based on the conclusions resulting from this thorough analysis of 100 selected *B. cereus* strains on biophysical characteristics, strain categories were screened on potential hazardous properties which could lead to intoxication if conditions are favorable. Potential hazardous characteristics of *B. cereus* strains selected from the environment, food and potential food poisoning cases (emetic and diarrhoeal) will be discussed in the following paragraphs.

5.1 Environmental strains

A positive correlation between minimum and maximum growth temperatures indicates psychrotolerant and mesophilic *B. cereus* strains are equally represented in the environment. However, as a majority of the environmental strains have a relatively low maximum growth temperature (< 48 °C), the correlation suggests that the environment contains relatively many psychrotolerant strains (having low minimum growth temperatures). This, in combination with the found negative correlation between minimum growth temperature and HBL-L2/B production makes environmental strains to be potentially hazardous if conditions are favorable. That is, the relatively many psychrotolerant strains in this category do not only grow well at low temperatures, these conditions are also optimal for enterotoxin production. If these results were to be generalized, although further research is needed before doing so, one could come to the following considerations. That is, keeping environmental strains at low temperatures (fridge) could still have hazardous consequences, *i.e.* growth of bacteria and, with that, optimal conditions for the production of enterotoxins. However, as enterotoxins are very unstable proteins they will probably readily be decomposed in food products and so any enterotoxins produced before consumption will presumably not form any hazard to public health. Still, outgrowth of vegetative cells in food products forms a potential hazard in itself. That is, environmental strains in this study have significantly low lag times compared to the other groups at in vivo conditions (37 °C/pH 5). And so, although environmental strains might be slow growers (psychrotolerant), a potential hazard could still occur when a lot of vegetative cells would enter the small intestine and instantly (after a short lag time) start producing high concentrations of enterotoxins.
On the other hand, environmental strains seem to have low times until first decimal reduction (d-value, not significant) and so thorough cooking might solve this problem.

5.2 Food strains

Food strains showed relatively little growth at 4 °C and low growth rates at 37 °C (*in vivo* conditions) with pH7. In addition, food strains have relatively high lag times at 37 °C. This indicates that good regulated fridge temperatures could prevent many intoxication problems stemming from *B. cereus* strains selected from food products.

5.3 Emetic strains

With minimum growth temperatures ≥ 10 °C and maximum growth temperatures ≥ 48 °C, emetic strains seem to be all mesophilic strains. In addition, germination was found to be low at 7 °C. This indicates that potential intoxication from emetic strains could be prevented, if kept at well-regulated fridge temperatures. However, if kept at room temperatures, emetic strains can grow relatively fast and, with that, produce the very stable emetic toxins. In that case, even cooking will not solve the problem as emetic strains seem to be associated with high decimal reduction times (*D*-values) and the toxins are heat stable. Still, as the estimated *D*-values lie well outside the assessment time of the heat resistant experiments, one should not bring these estimates into practice. *D*-values as estimated in this study can only be used for comparison reasons and, with that, we conclude that emetic strains have relatively high *D*-values.
Furthermore, emetic strains are associated with high growth rates at 37°C (pH7) and, therefore, form an increased hazard to human if food contaminated with emetic strains would be kept outside the fridge. In other words, emetic strains should be prevented from outgrowth at any time. Therefore, fast chilling after initially prepared food is of great importance as reheating will not help once emetic strains have grown in the food.

5.4 Diarrhoeal strains

Foods contaminated with diarrhoeal strains form an increased risk if not stored at temperatures below 7 °C. That is, a majority of the diarrhoeal strains were found to have a minimum growth temperature at 7 °C. Furthermore, diarrhoeal strains showed significantly high germination levels at 30 °C, which confirms their increased hazard to human if not handled properly already before consumption. As with the environmental strains, the formation of unstable enterotoxins before consumption does not evoke the problem as much as the outgrowth of vegetative cells in food products.

In summary,
preventing *Bacillus cereus* foodborne poisoning in Europe would primarily ask for:

- Well regulated fridge temperatures (< 7 °C) in order to prevent food poisoning from diarrhoeal strains.
- Food products having a non-neutral pH value as most of the 100 selected *B. cereus* strains showed instant growth at pH 7 independent of growth temperatures (24 °C and 37 °C).
- Proportioned chilling in order to rapidly lower the temperatures of precooked foods. This will prevent a fast outgrowth of emetic strains under these, for them, favorable conditions.

6. Discussion

Analysis of the 100 strain set reveals that biodiversity in *B. cereus* is large, both between and within groups. This has major consequences for future risk assessments concerning this foodborne pathogen (Pielaat et al., in preparation). Still, emetic strains seem to be specifically different from the other groups concerning the tested biophysical properties (*i.e.* growth temperatures, heat resistance, germination and estimated lag times and growth rates). Yet, to be able to generalize the outcomes of this study some drawbacks should be considered. That is, the variance in estimated lag times and growth rates among strain categories is assumed to be equal when performing an Analysis of Variance. Figures 17 to 23 show that this might not be true for certain pH/temperature combinations. In addition, parameters of the Baranyi and Roberts (1994) model could not be estimated with growth data of some strains. This is particularly critical for those strains which showed no growth within the time of the experiment. A lag time estimate of > 24 hours and a zero growth rate should have been estimated for these strains. This could make a large difference in the presented results. The growth data, as used in this study, actually ask for a more sophisticated statistical analysis, including a data dependent step-wise model selection procedure for parameter estimation. Furthermore, the initial strain selection has not been performed properly to actually identify statistically valid causal relationships between groups of *B. cereus* strains concerning tested biophysical characteristics. That is, the 100 strain set, selected from different laboratory cultures and used during experiments in this study, is not necessarily representative for *B. cereus* strains as found under natural circumstances. Statistical inference concerning the biodiversity of groups of *B. cereus* strains in general should be based on a random selection of strains from different sources, such as the environment, food products, patients, etc. instead of using laboratory strains that once were selected from different sources. Finally, at least 30 strains per group would be needed for a valid statistical analysis when the population characteristic under investigation is not necessarily assumed to have a Normal distribution (Central Limit Theorem). Future, in depth, analysis should account for these considerations.

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Appendix 1 Summary of raw data used to assess biodiversity in *Bacillus cereus*

Category	WSBC	Original code	Additional codes	Origin	Geographical location	Date of isolation	Sent by	Assigned (p)sychrotolerant and (m)esophylic	Min growth temp in°C (TUM)	Max growth temp in°C (TUM)	Foodpoisoning	Cytotoxicity emetic toxine (LMU)	Emetic toxin (TUM) PCR-800/NRPS and (UH-DACM) LC-MS	Diarrhoeal (LMU)	Hbl-PCR (INRA)	Titer HBL-L2 (LMU)	Antigen titer HBL-B (LMU)	CytK -PCR (INRA)	Nhe-PCR (INRA)	Antigen NHE-A (LMU)	Antigen titer NHE-B (LMU)	Estimated D-value from heat resistance data (90 C) (INRA)	Germination (7 C) (INRA)	Germination (30 C) (INRA)
food	10030	WSBC10030		pasteurized milk	Germany	1993	TUM	m	10	48	no	-	-	1316	-	-	-	-	+	+	4067	372	0.74	1.70
food	10204	WSBC10204		pasteurized milk	Germany	1992	TUM	p	4	n.g. at 42	no	-	-	-	+	-	-	+	+	+	33			
food	10286	WSBC10286		cream	Dairy G4	1994	TUM	m	10	48	no	-	-	909	+	-	-	-	+	+	1577	367	0.26	1.27
environment	10310	WSBC10310		soil	Weihenstephan, Germany	1997	TUM	m	>10	48	no	-	-	369	+	-	-	-	+	+	855			
food	10377	WSBC10377		raw milk	Germany	1995	TUM	p	4	48	no	-	-	143	+	-	-	+	+	+	349	54	2.41	0.87
food	10395	WSBC10395		raw milk	Germany	1995	TUM	m	>10	48	no	-	-	758	+	1004	329	+	+	+	1533	164	2.54	1.84
environment	10441	WSBC10441		soil	Thailand	1997	TUM	m	10	45	no	-	-	633	+	-	-	-	+	+	1578	64	2.54	3.36
food	10466	WSBC10466		red rice	Thailand	1997	TUM	p	7	48	no	-	-	690	-	-	-	+	+	+	1389	59	2.36	2.87
food	10483	WSBC10483		tobacco	India	1997	TUM	m	10	45	no	-	-	709	-	-	-	-	+	+	1484	56	0.82	3.83
food poisoning - emetic	10530	F4810/72	DSM4312, SMR-178	Vomit from cooked rice	USA or UK ???	1972	INRA/UHDAM	m	10	48	emetic syndrome	2000	+	1695	-	-	-	-	+	+	3958	763	0.10	1.92
food	10531	MHI 87		baby food	Germany/Munich	1993	LMU	m	>10	48	no	1000	+	962	+	-	-	-	+	+	2070	318	0.28	1.44
food poisoning - emetic	10536	MHI 1305		Indian rice dish	Germany/Passau	2001	LMU	m	>10	48	yes	232	+	1429	+	-	-	-	+	+	2419	672	0.29	1.17
food poisoning - emetic	10537	NC7401		vomit	Japan	1994	UHDAM	m	>10	48	emetic syndrome	769	+	833	-	-	-	-	+	+	3432	612	0.22	1.95
environment	10538	UHDAM NS-115		Spruce three	Finland	1988	UHDAM	m	>10	48	no	25	+	-	-	-	-	-	+	-	-	341	0.25	1.45
food poisoning - emetic	10542	F3080B/87		Chicken Korma & rice	UK (PHLS)	1987	INRA,UHDAM	m	>10	48	emetic syndrome	2500	+	1471	-	-	-	-	+	+	4483	372	0.20	2.03
food poisoning - emetic	10544	F3351/87		Faeces, fried rice	UK (PHLS)	1987	INRA	m	10	48	emetic syndrome	1350	+	1250	-	-	-	-	+	+	2572	537	0.23	2.47
food	10550	INRA 1		Zucchini purée	Normandie, France	Aug-98	INRA Avignon	p	4	48		-	-	-	+	-	-	-	+	-	-	60	2.87	0.80
food poisoning - diarrhoeal	10552	NVH 0075-95	NVH25	Stew with vegetables,foodpoisoning	Norway	1995	PEG	m	>10	48	Food poisoning/ diarrhoeal syndrom	-	-	1282	-	-	-	-	+	+	3325	185	0.26	1.37
food poisoning - diarrhoeal	10553	NVH 0230-00	NVH26	Buffet dinner, mushroom stew, foodpoisoning, oubreak	Norway	2000	PEG	p	7	48	Food poisoning/ diarrhoeal syndrom	-	-	769	+	372	439	+	+	+	2076	92	3.40	3.63
food poisoning - diarrhoeal	10554	NVH 0500-00	NVH27	Potatoes in cream sauce, foodpoisoning, outbreak	Norway	2000	PEG	p	7	48	Food poisoning/ diarrhoeal syndrom	-	-	2500	-	-	-	-	+	+	5891	201	1.93	4.02

Category	WSBC	Original code	Additional codes	Origin	Geographical location	Date of isolation	Sent by	Assigned (p)sychrotolerant and (m)esophylic	Min growth temp in°C (TUM)	Max growth temp in°C (TUM)	Foodpoisoning	Cytotoxicity emetic toxine (LMU)	Emetic toxin (TUM) PCR-800/NRPS and (UH-DACM) LC-MS	Diarrhoeal (LMU)	Hbl-PCR (INRA)	Titer HBL-L2 (LMU)	Antigen titer HBL-B (LMU)	CytK -PCR (INRA)	Nhe-PCR (INRA)	Antigen NHE-A (LMU)	Antigen titer NHE-B (LMU)	Estimated D-value from heat resistance data (90 C) (INRA)	Germination (7 C) (INRA)	Germination (30 C) (INRA)
food poisoning - diarrhoeal	10555	NVH 0861-00	NVH28	Soft ice (ice-cream)	Norway	2000	PEG	p	7	45	Food poisoning/ diarrhoeal syndrom	-	-	3030	-	-	-	-	+	+	7757	54	1.15	2.32
food poisoning - diarrhoeal	10556	NVH 1230-88	NVH29	Oriental stew	Norway	1988	PEG	p	7	45	Food poisoning, diarrhoeal syndrome	-	-	641	+	1011	633	+	+	+	1924	49	1.10	3.59
food poisoning - diarrhoeal	10557	NVH 1519-00	NVH30	Stew with deermeet	Norway	2000	PEG	m	>10	48	diarrhoeal syndrome	-	-	1818	-	-	-	-	+	+	4181	93	0.39	2.30
food poisoning - diarrhoeal	10558	NVH 1651-00	NVH31	Caramel pudding	Norway	2000	PEG	p	7	45	Food poisoning, diarrhoeal syndrome	-	-	588	+	179	147	+	+	+	1935	63	1.08	2.06
food poisoning - diarrhoeal	10559	NVH 0391-98	NVH32	Vegetable pure	France	1998	PEG	p	7	45	Food poisoning/ diarrhoeal syndrom	-	-	335	-	-	-	+	-	-	-	60	0.70	2.44
food poisoning - diarrhoeal	10560	98HMPL63		cooked salsify	France	2000	INRA	p	7	45	diarrhoeal syndrome	-	-	641	+	654	530	+	+	+	2207	45	2.03	2.43
food	10561	INRA A3		Starch	Normandie, France	Aug-98	INRA Avignon	m	>10	45		-	-	437	+	150	116	+	+	+	1623	208	1.48	2.45
food	10563	INRA C3		Pasteurized carrot	Vaucluse, France	Dec-96	INRA Avignon	p	7	45		-	-	602	+	346	329	+	+	+	2545	46	2.52	2.55
food	10564	INRA C24		Pasteurized carrot	Vaucluse, France	Dec-96	INRA Avignon	m	>10	48		-	-	1053	-	-	-	-	+	+	3082	152	0.25	2.32
food poisoning - diarrhoeal	10565	FH3502/72	DSM 2301	Foodborne outbreak		1972	INRA	p	7	48	diarrhoeal syndrome	-	-	-	-	-	-	-	+	+	40	300	2.32	4.44
food poisoning - diarrhoeal	10566	F837/76	DSM 4222	Foodborne ourbreak	UK (PHLS)	1976	INRA	m	10	48	diarrhoeal syndrome	-	-	1563	+	556	229	-	+	+	3068	142	3.17	3.76
food poisoning - diarrhoeal	10568	NRS 404	DSM 8438	Foodborne ourbreak (clinica isolate)			INRA	p	7	48	diarrhoeal syndrome	-	-	690	-	-	-	+	+	+	2028	53	1.69	2.29
food	10571	SDA MA57	EU13	raw milk	Sweden	1997	SDA	p	7	48		-	-	59	+	-	-	-	+	-	173	42	2.22	0.67
food	10574	SDA KA96	EU16	raw milk	Sweden	1997	SDA	m	10	45		-	-	935	+	1112	963	+	+	+	3932	41	2.52	2.80
food	10576	SDA GR177	EU18	raw milk	Sweden	1998	SDA	m	10	48		128	+	862	+	-	-	-	+	+	3093	489	0.07	2.16
food poisoning - diarrhoeal	10581	F2081A/98		Cooked chicken		1998	INRA	p	7	48	diarrhoeal syndrome	-	-	571	+	231	200	+	+	+	1447	29	2.00	2.91
food poisoning - diarrhoeal	10583	F2085/98		Cooked rice		1998	INRA	m	10	48	diarrhoeal syndrome	-	-	1031	-	-	-	+	+	+	2241	52	2.10	2.67
food poisoning - diarrhoeal	10590	F3371/93		Chinese takeaway chop suey	UK (PHLS)	1993	INRA	p	7	48	diarrhoea, vomit, fever	-	-	392	+	187	283	+	+	+	1338	47	3.94	3.47

Category	WSBC	Original code	Additional codes	Origin	Geographical location	Date of isolation	Sent by	Assigned (p)sychrotolerant and (m)esophylic	Min growth temp in°C (TUM)	Max growth temp in°C (TUM)	Foodpoisoning	Cytotoxicity emetic toxine (LMU)	Emetic toxin (TUM) PCR-800/NRPS and (UH-DACM) LC-MS	Diarrhoeal (LMU)	Hbl-PCR (INRA)	Titer HBL-L2 (LMU)	Antigen titer HBL-B (LMU)	CytK -PCR (INRA)	Nhe-PCR (INRA)	Antigen NHE-A (LMU)	Antigen titer NHE-B (LMU)	Estimated D-value from heat resistance data (90 C) (INRA)	Germination (7 C) (INRA)	Germination (30 C) (INRA)
food poisoning - diarrhoeal	10591	F3453/94		Wedding reception rice dish	UK (PHLS)	1994	INRA	m	10	45	diarrhoea, vomit	-	-	400	+	18	16	+	+	+	1519	115	0.18	1.91
food poisoning - diarrhoeal	10593	F352/90		Chow Mein		1990	INRA	m	10	45	diarrhoeal syndrome	-	-	1111	+	485	1686	+	+	+	3187	40	1.74	3.64
food poisoning - diarrhoeal	10605	F4430/73	DSM 4384, B4ac	Pea soup	Belgium	1973	INRA	p	7	45	diarrhoeal syndrome	-	-	633	+	889	406	+	+	+	2456	49	0.46	0.70
food poisoning - diarrhoeal	10608	F4433/73		Meat loaf	USA	1973	INRA	p	7	45	diarrhoeal syndrome	-	-	427	+	810	719	+	+	+	2470	63	1.78	2.98
food poisoning - diarrhoeal	10613	F528/94		Chinese restaurant beef&chow mein&rice	UK (PHLS)	1994	INRA	p	7	45	vomit, diarrhoea, fever	-	-	242	+	650	711	-	+	+	1340	40	2.88	2.00
food	10616	INRA I20		Leek (cooked foods)	Market, France	Jan-91	ADRIA, France	p	4	45		-	-	769	+	-	-	-	+	+	2077	48	1.72	0.62
food	10617	INRA I21		Carrot (cooked foods)	Market, France	May-91	ADRIA, France	p	7	45		-	-	-	+	-	-	-	+	-	-	50	2.00	1.11
food poisoning - diarrhoeal	10618	IH41064		Faeces (food poisoning)	Finnland	1998	INRA	p	7	45	diarrhoea	-	-	323	+	405	353	+	+	+	1182	61	2.49	3.14
food poisoning - emetic	10619	IH41385		Dialysis liquid (connected to illness)	Finnland	1998	INRA	m	>10	48	emetic syndrome	16	+	820	-	-	-	-	+	+	3752	917	0.21	2.03
food poisoning - diarrhoeal	10624	NVH 200	97	Meat dish with rice	Denmark	1996	INRA	p	7	48	diarrhoeal syndrome	-	-	1389	-	-	-	+	+	+	5629	253	1.76	4.52
food poisoning - diarrhoeal	10629	RIVM BC 63	9.1 T1.1	human feces		2001	RIVM	p	7	48	yes	-	-	418	+	-	-	-	+	+	1700	260	0.32	1.79
food poisoning - emetic	10630	RIVM BC 67	9.1 T2.1	human feces		2001	RIVM	m	10	48	yes	2500	+	1667	+	-	-	+	+	+	4260	270	1.32	1.79
food poisoning - diarrhoeal	10633	RIVM BC 90	PAL 5	human feces		1999	RIVM	m	>10	48	yes	-	-	-	+	-	-	-	+	+	267	162	0.23	0.64
environment	10635	INRA SZ		soil	Normandie, France	Aug-98	INRA Avignon	p	7	45		-	-	435	+	254	329	-	+	+	1970	61	2.09	1.07
environment	10643	SDA 1R72		soil	FarmU, east coast, Sweden	1994	SDA	p	4	45		-	-	21	-	-	-	-	+	+	123	99	0.69	1.73
environment	10648	SDA 1R177		manure	FarmU, east coast, Sweden	1994	SDA	p	4	45		-	-	1124	+	394	393	-	+	+	1711	75	2.48	1.88
environment	10649	SDA 1R183		manure	FarmU, east coast, Sweden	1994	SDA	m	10	45		-	-	568	+	21	41	+	+	+	1071	52	2.05	1.90
environment	10685	SDA NFFE640		air	Dairy1, south, Norway	1997	SDA	p	4	45		-	-	694	+	266	369	-	+	+	1352	74	2.00	1.46
environment	10688	SDA NFFE664		water hose	Dairy1, south, Norway	1997	SDA	p	4	45		-	-	813	+	391	385	-	+	+	1468	78	2.20	1.47

Category	WSBC	Original code	Additional codes	Origin	Geographical location	Date of isolation	Sent by	Assigned (p)sychrotolerant and (m)esophylic	Min growth temp in°C (TUM)	Max growth temp in°C (TUM)	Foodpoisoning	Cytotoxicity emetic toxine (LMU)	Emetic toxin (TUM) PCR-800/NRPS and (UH-DACM) LC-MS	Diarrhoeal (LMU)	Hbl-PCR (INRA)	Titer HBL-L2 (LMU)	Antigen titer HBL-B (LMU)	CytK -PCR (INRA)	Nhe-PCR (INRA)	Antigen NHE-A (LMU)	Antigen titer NHE-B (LMU)	Estimated D-value from heat resistance data (90 C) (INRA)	Germination (7 C) (INRA)	Germination (30 C) (INRA)
environment	10690	SDA NFFE647		water	Dairy1, south, Norway	1997	SDA	p	4	42		-	-	758	+	329	367	-	+	+	1542	68	1.19	0.43
food poisoning - diarrhoeal	10702	MHI 203		pasta	Germany/Munich	1998	LMU	p	7	45	yes	-	-	588	+	184	348	-	+	+	998	70	0.82	2.39
food	10705	MHI 13		baby food	Germany/Munich	1993	LMU	m	10	45	no	-	-	862	-	-	-	-	+	+	2012	796	1.90	1.54
food	10708	MHI 32		baby food	Germany/Munich	1993	LMU	p	7	45	no	-	-	1724	+	367	128	+	+	+	2792	396	0.48	2.09
food	10709	MHI 124		baby food	Germany/Munich	1993	LMU	m	10	48	no	-	-	322	-	-	-	+	+	+	755	140	0.75	2.32
food	10718	INRA C57		Potato purée	Vaucluse, France	Sep-96	INRA Avignon	p	7	48		-	-	212	+	92	92	+	+	+	527	49	3.01	1.35
food	10730	INRA PA		Milk proteins	Normandie, France	Aug-98	INRA Avignon	m	>10	48		-	-	-	-	-	-	+	+	+	764	402	0.42	1.46
food poisoning - diarrhoeal	10735	UHDAM B217		human feces (food poisoning)	Finland	2000	UHDAM	p	7	48	yes	-	-	1111	-	-	-	+	+	+	3131	131	0.55	2.11
food poisoning - diarrhoeal	10736	UHDAM B154		cake (food poisoning)	Finland	2000	UHDAM	p	7	48	yes	-	-	1587	+	210	353	+	+	+	1747	48	4.04	3.82
food	10737	UHDAM B102		meat pie	Finland	2000	UHDAM	m	10	48	?	-	-	229	+	-	-	-	+	+	452	92	1.51	1.76
food poisoning - diarrhoeal	10738	UHDAM B106		potatoe flour (quality control sample)	Finland	2000	UHDAM	p	7	48	yes	-	-	1299	-	-	-	-	+	+	1891	301	2.14	3.16
food poisoning - emetic	10740	UHDAM ML127		pasta	Finland	2000	UHDAM	m	10	48	yes	5120	+	-	+	-	-	-	+	+	-	318	0.48	1.12
food poisoning - emetic	10742	UHDAM B315		cake	Finland	2000	UHDAM	m	10	48	yes	143	+	-	-	-	-	-	+	+	-	129	0.49	0.72
food	10744	UHDAM 1IFI(1)		infant food	Finland	2002	UHDAM	m	>10	48	no	1429	+	37	-	-	-	-	+	+	1948	68	0.60	2.12
food	10746	UHDAM 1IFI(3)		infant food	Finland	2002	UHDAM	m	>10	48	no	3333	+	485	-	-	-	-	+	+	1339	371	0.41	1.09
food	10747	UHDAM 1IFI(13)		infant food	Finland	2002	UHDAM	m	10	48	no	-	-	455	+	-	-	-	+	+	1365	152	0.60	0.12
environment	10756	UHDAM 3/pkl		filler material from hospital	Finland		UHDAM	m	>10	48		61	+	1786	+	-	-	-	+	+	2904	372	0.20	2.45
food poisoning - diarrhoeal	10760	NVH 0309-98	NVH386	Rice	Norway	1998	PEG	m	10	45	Food poisoning	-	-	256	+	9	-	-	+	+	717	254	1.62	1.79
food poisoning - diarrhoeal	10761	NVH 0674-98	NVH390	Scrambled eggs	Norway	1998	PEG	p	7	45	Food poisoning	-	-	926	+	-	-	-	+	+	1483	161	3.29	3.31
food poisoning - diarrhoeal	10762	NVH 1104-98	NVH395	Fish-soup	Norway	1998	PEG	p	7	42	Food poisoning	-	-	667	+	98	169	-	+	+	2271	74	3.00	3.90
food poisoning - diarrhoeal	10763	NVH 1105-98	NVH396	Topping on steak	Norway	1998	PEG	p	7	45	Food poisoning	-	-	521	+	-	-	+	+	+	992	817	0.79	1.39
food poisoning - diarrhoeal	10764	NVH 0165-99	NVH424	Deer-steak	Norway	1999	PEG	p	7	48	Food poisoning	-	-	233	+	-	-	-	+	+	336	69	1.84	1.57
food poisoning - diarrhoeal	10766	NVH 0597-99	NVH430	Mixed spices	Norway	1999	PEG	p	7	48	Food poisoning	-	-	442	-	-	-	+	+	+	918	41	1.79	2.44

Category	WSBC	Original code	Additional codes	Origin	Geographical location	Date of isolation	Sent by	Assigned (p)sychrotolerant and (m)esophylic	Min growth temp in°C (TUM)	Max growth temp in°C (TUM)	Foodpoisoning	Cytotoxicity emetic toxine (LMU)	Emetic toxin (TUM) PCR-800/NRPS and (UH-DACM) LC-MS	Diarrhoeal (LMU)	Hbl-PCR (INRA)	Titer HBL-L2 (LMU)	Antigen titer HBL-B (LMU)	CytK -PCR (INRA)	Nhe-PCR (INRA)	Antigen NHE-A (LMU)	Antigen titer NHE-B (LMU)	Estimated D-value from heat resistance data (90 C) (INRA)	Germination (7 C) (INRA)	Germination (30 C) (INRA)
food poisoning - diarrhoeal	10770	NVH 1518-99	NVH451	Soft ice (ice-cream)	Norway	1999	PEG	m	>10	48	Food poisoning	-	-	238	+	-	-	+	+	+	546	74	1.23	3.17
food poisoning - diarrhoeal	10772	NVH 0226-00	NVH463	Turkey	Norway	2000	PEG	p	7	45	Food poisoning	-	-	369	+	200	260	+	+	+	569	55	0.71	2.02
food poisoning - diarrhoeal	10774	NVH 0784-00	NVH493	Ground beef	Norway	2000	PEG	p	7	45	Food poisoning	-	-	699	+	-	-	+	+	+	787	92	2.97	3.81
food poisoning - diarrhoeal	10778	NVH 141/1-01	NVH584	Vegetarian pasta	Norway	2001	PEG	p	7	45	Food poisoning	-	-	226	+	65	88	-	+	+	343	79	2.43	3.69
food poisoning - diarrhoeal	10779	NVH 0154-01	NVH588	Figs	Norway	2001	PEG	m	10	45	Food poisoning	-	-	316	+	16	44	+	+	+	372	48	2.20	3.01
food	10788	NVH 449	NVH449	Spices	Norway	1999	PEG	m	10	45		-	-	289	+	397	135	-	+	+	1444	178	2.51	4.23
food	10791	NVH 506	NVH506	Spices	Norway	2000	PEG	m	10	52		-	-	42	-	-	-	-	+	+	-	328	0.22	0.02
food	10797	NVH 445	NVH445	Meat	Norway	1999	PEG	m	10	48		-	-	571	+	-	-	+	+	+	1063	241	0.46	1.70
environment	10801	NVH 460	NVH460	Equipment	Norway	2000	PEG	p	7	48		-	-	602	+	141	372	+	+	+	1386	68	1.34	3.55
environment	10805	NVH 512	NVH512	Equipment	Norway	2000	PEG	p	7	45		-	-	885	+	-	-	-	+	+	1475	66	2.89	3.02
environment	10811	NVH 655	NVH655	River water	Norway	2002	PEG	p	7	42		-	-	385	+	298	411	+	+	+	1577	44	3.14	2.47
food poisoning - diarrhoeal	10820	RIVM BC 122	9902550	patients feces		1999	RIVM	m	10	45		-	-	-	-	-	-	-	+	+	-	81	2.35	2.16
food poisoning - emetic	10821	RIVM BC 124	9902632	patients feces		1999	RIVM	m	10	48		1429	+	855	+	-	-	+	+	+	1070	369	0.24	1.19
food	10834	RIVM BC 934	KAL 734	lettuce		2002	RIVM	m	>10	48		-	-	348	+	41	77	-	+	+	492	113	2.47	2.65
food	10836	RIVM BC 938	KAL 738	lamb's lettuce		2002	RIVM	p	7	48		-	-	408	+	-	-	-	+	+	601	64	2.99	3.22
food	10843	RIVM BC 379	KAL179	chicken		2001	RIVM	m	>10	48		76	+	129	-	-	-	-	+	+	274	289	3.72	1.15
food	10845	RIVM BC 485	KAL 285	chicken ragout		2001	RIVM	p	7	45		-	-	382	+	-	-	+	+	+	698	140	1.92	2.84
food	10850	RIVM BC 964	KAL 764	kebab		2002	RIVM	p	7	42		-	-	752	-	-	-	-	+	+	1293	115	2.10	3.01
environment	10854	UHDAM TSP9		Paper board	Finland	1996	UHDAM	m	10	45	no	-	-	33	+	5	-	-	+	+	266	66	2.09	2.57
food	10871	SDA GR285		raw milk	Sweden	1998		p	7	48		-	-	1283	+	71	41	-	+	+	1170	105	1.00	2.54
Count	100	100	44	100	85	98	99	100	100	100	67	100	100	100	100	100	100	100	100	100	100	98	98	98