Development of a time-to-detect growth model for heat-treated
Bacillus cereus spores

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Abstract

The microbiological safety and quality of Refrigerated Processed Foods of Extended Durability (REPFEDs) relies on a combination of mild heat treatment and refrigeration, sometimes in combination with other inhibitory agents that are ineffective when used alone. In this context, a predictive model describing the time-to-detect growth (measured by turbidimetry) of psychrotrophic Bacillus cereus spores submitted to various combinations of pH, water activity (aw), heat treatment and storage temperature was developed. As the inoculum was high, the time-to-detect growth was set as the longest time where none, or only one, of the 8 replicated wells showed growth. In the no-growth area (211 combinations) the time-to-detect growth was set as longer than the time where the experiment was stopped (60 days or more) and analysed as a censored response. The factors of variation were heat-treatment intensity (85 °C, 87 °C and 90 °C in a time range of 1 to 38 min), storage temperature (8–30 °C), pH (5.2–6.4) and aw (0.973–0.995). Two different strains were analysed. The model had a Gamma multiplicative structure; it was solved by Bayesian inference with informative prior distributions. To be implemented in a decision tool, for instance to calculate the process and formulation conditions required to achieve a given detection time, each Gamma term had some constraints: they had to be monotonous, continuous and algebraically simple mathematical functions (i.e. having analytical solution).

Overall, the cumulative effect of various stressful conditions (pasteurisation process, low temperature, and low pH) enables to extend the time-to-detect growth up to 60 days or more, whereas the heat-treatment on its own did not have a similar effect. For example, with the most heat resistant strain (strain 1, FF140), for a product at aw 0.99, stored at 10 °C, heat-treated at 90 °C for 10 min, a time-to-detect growth of 2 days was expected when the pH equalled 6.5. Under the same conditions, if the pH was reduced to 5.8, the time-to-detect growth was predicted to be 11 days (and 33 days at pH 5.5). After a pasteurisation at 90 °C for 10 min, for a product kept at 10 °C, combinations of pH and aw such as pH 6.0–aw 0.97, pH 5.7–aw 0.98 or pH 5.5–aw 0.99 were predicted to extend the time-to-detect growth up to 30 days. The developed model is a useful tool for REPFED producers to guarantee the safety of their products towards psychrotrophic B. cereus.

1. Introduction

Refrigerated Processed Foods of Extended Durability (REPFEDs) are becoming increasingly popular following consumer demand for more convenient, less preserved chilled food products with longer

shelf-lives and higher organoleptic quality (Carlin et al., 2004; Del Torre et al., 2004). The shelf-life of REPEDs is generally in a range of two/three up to five/six weeks depending on the production process and product formulation. In REPEDs, it is generally agreed that a temperature of 90 °C for 10 min will deliver a 6D inactivation of non-proteolytic Clostridium botulinum and this is a commonly used performance standard for the heat processing of chilled foods (Gould, 1999). However, spores of psychrotrophic Bacillus cereus might survive a pasteurisation of 90 °C for 10 min and germinate, after which the
vegetative cells can grow up to a hazardous levels ($>10^5$ CFU/g). The extent to which $B.$ cereus may pose a safety risk within a REPFED will depend on many factors including: spore prevalence and concentration in raw materials, heat treatment, heat resistance of spores, product formulation and supply-chain storage temperatures (Membré and Lambert, 2008). In particular, the effect of the heat treatment and product formulation on the subsequent lag time of surviving spores can have a significant impact on the safe shelf-life (Barker et al., 2005; Faille et al., 1997; Laurent et al., 1999; Martínez et al., 2007).

To our best knowledge, there is no off-the-shelf predictive model describing the combination of thermal processing and product formulation on $B.$ cereus spore lag time. Likewise, there is not much data publicly available showing the effect of both heat-treatment and formulation conditions on $B.$ cereus spores. When searching in ComBase (Baranyi and Tamplin, 2004) for log count growth curves (selected criteria: $B.$ cereus, culture medium, temperature below or equal $20 \, ^\circ C$), the lag time values were relatively short even under stressful conditions, for instance, at temperature $7 \, ^\circ C$ and pH 5.5, lag times were less than one week (record B130.59 and B130.60, data from Campden and Chorleywood Food Research Association).

The objective of this study was to develop a mathematical model quantitatively assessing the effect of the factors related to the production process (heat treatment), the product formulation (pH and water activity ($a_w$)) and the environment (chilled storage temperature, or alternatively ambient temperature during product preparation) on the lag time of heat-treated $B.$ cereus spores. However the spore lag time, i.e. the sum of time required to have spore germination and out-growth, was not measured directly. Instead, a set of growth/no-growth data previously generated at Ghent University (Daelman et al., 2013) was reanalysed to extract the time before detecting growth. The limit of detection of the turbidimetry method used to generate the data was 1.310^6 CFU/ml, and consequently the spore inoculum was deliberately high ($10^5$ to $10^6$) to achieve the detection limit quickly. Obviously, strictly speaking, the time before detecting growth (hereafter referred to as ‘detection time’) is always longer than the spore lag time. However, with a high inoculum, the outgrowth time is short in comparison to the lag time and the detection time is relatively close to the spore lag time. A total of 434 data was analysed. To enable a further use of the model in determining the various conditions of formulation and processing that guarantee detection times longer than a desired value (e.g. 30 days or 50 days), the mathematical model chosen had three constraints: i) to be continuous in the model domain of use, ii) to be based on monotonous functions for each factor, iii) to remain algebraically simple. These constraints enable to directly determine a single heat-treatment process suitable to deliver a given detection time for a specific formulation (pH and $a_w$), or vice-versa (one formulation for a specific process). This application was considered as highly valuable for further process and formulation optimization, either carried out with the time-to-detect growth model run on its own, or incorporated in a more comprehensive farm-to-fork risk assessment model.

2. Materials and methods

2.1. The dataset

The experimental protocol is described in a publication by Daelman et al. (2013). The experiments were performed using 8 wells from a microtitre plate reader for each condition. When growth occurred in at least 2 wells at time t, the detection time was defined at t − 1 (longest time where no growth was observed in 7 wells), this was an observed detection time. On the other hand, when no visible growth was observed in any of the 8 wells, the detection time was considered to be longer than the time when the experiment was stopped (60 days or more), and analysed as a right-censored data.

The detection time of two strains of $B.$ cereus isolated from REPFEDs, strain 1 (FF140 isolated from béchamel sauce) and strain 2 (FF355 isolated from carrots) was studied as a function of the factors heat treatment (time and temperature), pH, $a_w$ and storage temperature. Spores of strain 1 have a $D_{90 \, ^\circ C}$-value of 90.9 min, while spores of strain 2 have a $D_{90 \, ^\circ C}$-value of 17.9 min. Both strains have similar z-values of 9.6 °C and 9.5 °C, for strains 1 and 2, respectively (Daelman et al., 2013). In Table 1, the experimental conditions are provided, for each factor and each strain. From the 434 data points collected on $B.$ cereus spores, 223 corresponded to observed detection time values and 211 to censored data. Among the 223 observed values, detection times varied within a range of 0.2 to 56.6 days. A set of 26 data presented detection times lower than or equal to 1 day while the factors heat treatment, pH, $a_w$ and storage temperature were not altogether at their optimal values (Table 2). For example, detection times of 1 day were obtained at storage temperature of 10 °C and pH 5.6 when the heat-treatment condition was mild (85 °C or 87 °C for 1 min).

The initial inoculum of the two strains before heat-treatment was chosen so that after thermal inactivation, a heat-treated spore concentration of $10^5$ to $10^6$ CFU/ml was obtained for strain 1 and strain 2. Since the heat treatment was applied after spore inoculation, the spores were in the same medium, and the same pH and $a_w$ conditions, during the whole experiment (from initial inoculation to 60 days or more).

2.2. The time-to-detect growth model applied to heat-treated spores

The model used to predict the detection time (time from inoculation to first growth observation) of heat-injured spores of $B.$ cereus has been adapted from the set of equations developed for predicting non-proteolytic $C.$ botulinum spore lag time (Membré, 2012). The general framework follows the Gamma concept originally suggested for microbial growth rate (Wijtzes et al., 1998; Zwietering, 2002).

The detection time was described by a general equation, with multiplicative terms (Eq. (1)). The natural logarithm transformation of the response, i.e. of the detection time value, was chosen to stabilise the variance.

$$\ln(lag + 1) = \alpha_s \times \prod_{i}^{k} \gamma_i - 1 + \varepsilon$$

(1)

In Eq. (1), “lag” corresponded to the time-to-detect growth, explained for a large part by the spore lag phase but included as well vegetative cell growth up to a detectable level. There were four $\gamma_i$ terms corresponding to the four factors ($k = 4$): storage temperature ($S_T$), pH, $a_w$ and heat treatment ($HT_t$, itself split into heat-treatment time, $HT_t$ and heat-treatment temperature, $HT_T$). The index “s” corresponded to the strain ($s = 1$ or $2$). The residual error term, $\varepsilon$, was assumed to be normally distributed: $\varepsilon \sim N(0, \sigma_\varepsilon^2)$. The left hand side of Eq. (1) was written as $\ln(lag + 1)$ to enable the logarithm transformation even with detection time values reported as zero.

There are two main modifications in comparison with a Gamma structure. The first one lies in the addition of a term “−1” in Eq. (1). Indeed, with this “−1” term, the right hand side of Eq. (1) could be negative, particularly when the factors $S_T$, pH, $a_w$, $HT_t$ and $HT_T$ were equal, or close to their optimal values. Likewise, this additional term “−1” enabled to get a detection time nil (in Eq. (1), $\ln(lag + 1) = 0$ is equivalent to lag = 0) when the factors were not at their optimal values and meant that the coefficient $\alpha_s$ did not correspond to the minimal detection time observed. However, this mathematical parameterization was chosen to include in the model all the data reporting very short detection times (detection time equals to 1 day or less) at sub-optimal conditions (Table 2). Overall, the parameterization chosen here meant that the Gamma model sensu stricto over-estimated the detection time and had to be moderated (Eq. (2)).

$$\text{lag} + 1 = \exp\left(\alpha_s \times \prod_{i}^{k} \gamma_i \right) / \exp(1).$$

(2)
Table 1
Dataset used to build the model: heat-treatment (HT) temperature and time, *B. cereus* strains, status (censored data or lag times), aw, pH and storage temperature conditions.

<table>
<thead>
<tr>
<th>HT temperature value</th>
<th>Strain Status and range of heat treatment time [min-max]</th>
<th>Formulation and environment factors [min-max]</th>
</tr>
</thead>
<tbody>
<tr>
<td>212 data collected at 85 °C</td>
<td>89 data on strain 2</td>
<td>33 data (lag time value) for HT time of [1–38] min aw [0.973–0.995] pH [5.2–6.4] Storage temperature [8.0–10.0]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>56 censored data (&gt;60 days) for HT time of [1–38] min aw [0.973–0.995] pH [5.2–6.0]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>123 data on strain 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>119 data collected at 87 °C</td>
<td>59 data on strain 1</td>
<td>34 data (lag time value) for HT time of [1–38] min aw [0.973–0.995] pH [5.2–6.4] Storage temperature [8.0–10.0]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25 censored data (&gt;60 days) for HT time of [1–38] min aw [0.973–0.995] pH [5.2–5.6]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60 data on strain 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>103 data collected at 90 °C</td>
<td>47 data on strain 1</td>
<td>38 data (lag time value) for HT time of [1–38] min aw [0.973–0.995] pH [5.2–6.4] Storage temperature [8.0–10.0]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>56 data on strain 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Moreover, the model parameterization (Eq. (1)) means that the model is applicable only to detection time data obtained in days, it would have been different with data expressed for instance in hours.

The second difference with the lag model previously developed for bacterial spores (Membré, 2012) is a conditional effect of heat-treatment to the pH: the heat-treatment extended the detection time if and only if the pH was lower than its optimal (Eq. (3)). This assumption was made based on the conclusion of the study done by Daelman et al. (2013) with the same two strains: the authors highlighted a difference between the two strains in both minimal aw and minimal pH.

Concluding the two strains, the model was built with the following assumptions: the residual error, ε, was the same whatever the strain. This error encompassed experimental error, model adjustment error and natural variability in spore response. On the other hand, the strain response to a combination of stress (low storage temperature, low pH, low aw) was considered as dependent on the strain. This assumption was also based on the study done by Daelman et al. (2013), the authors highlighted a difference between the two strains in both minimal aw and minimal pH.

$$
\begin{align*}
\prod_{k} \gamma_{k} &= f_{1}^{(1)}(\text{StT}) \times f_{2}^{(2)}(\text{pH}) \times f_{3}^{(3)}(a_{w}) \times f_{4}^{(4)}(\text{HT}) & \text{if } pH \leq pH_{\text{opt}} \\
\prod_{k} \gamma_{k} &= f_{1}^{(1)}(\text{StT}) \times f_{2}^{(2)}(\text{pH}) \times f_{3}^{(3)}(a_{w}) & \text{if } pH > pH_{\text{opt}}
\end{align*}
$$

(3)
The effect of the four factors was expressed mathematically by a monotonous function as indicated below (Eqs. (4)–(10)).

\[ f_{11}(ST) = \begin{cases} \frac{1}{ST - T_{\text{min}}} & \text{for } ST \leq T_{\text{min}} \\ \frac{1}{ST - T_{\text{opt}}} & \text{for } T_{\text{min}} < ST \leq T_{\text{opt}} \\ \frac{1}{ST - T_{\text{opt}}} & \text{for } ST > T_{\text{opt}} \end{cases} \]

\[ f_{21}(pH) = \begin{cases} \frac{1}{pH - pH_{\text{opt}}} & \text{for } pH \leq pH_{\text{min}} \\ \frac{1}{pH - pH_{\text{min}}} & \text{for } pH_{\text{min}} < pH \leq pH_{\text{opt}} \end{cases} \]

The effect of \( a_w \) on the detection time was assessed firstly through a general pattern, including a specific effect of \( a_w \) on strain 1 (\( f_{31}(a_w) \)) and strain 2 (\( f_{32}(a_w) \)) as summarised in Eq. (6).

\[ f_{31}(a_w) = \begin{cases} \frac{a_w - a_{w_{\text{min}}}}{a_{w_{\text{max}}} - a_{w_{\text{min}}}} & \text{for } a_{w_{\text{min}}} \leq a_w \leq a_{w_{\text{opt}}} \\ \frac{1}{a_{w_{\text{max}}} - a_{w_{\text{min}}}} & \text{for } a_{w_{\text{min}}} < a_w < a_{w_{\text{opt}}} \end{cases} \]

However, the effect of \( a_w \) on strain 2 was not established, i.e. there was no change in the deviance information criterion, nor in the residual model error when a model with or without \( f_{32}(a_w) \) term. Consequently, it was decided to simplify the model as follows (Eq. (7)):

\[ f_{32}(a_w) = \begin{cases} \frac{a_w - a_{w_{\text{min}}}}{a_{w_{\text{max}}} - a_{w_{\text{min}}}} & \text{for } a_{w_{\text{min}}} \leq a_w \leq a_{w_{\text{opt}}} \\ \frac{1}{a_{w_{\text{max}}} - a_{w_{\text{min}}}} & \text{for } a_{w_{\text{min}}} < a_w < a_{w_{\text{opt}}} \end{cases} \]

The heat-treatment effect was split into heat-treatment time, \( HT \), and heat-treatment temperature, \( HT_T \) (Eqs. (8)–(10)). This effect was considered as independent of the strains.

\[ f_{41}(HT) = f_{41}(HT_T) \times f_{41}(HT_T) \]

\[ f_{41}(HT_T) = \begin{cases} \frac{HT_{\text{max}} - HT_{\text{opt}}}{HT_{\text{max}} - HT_{\text{opt}}} & \text{for } HT_T \geq HT_{\text{max}} \\ \frac{1}{HT_{\text{max}} - HT_{\text{opt}}} & \text{for } HT_{\text{opt}} \leq HT_T < HT_{\text{max}} \\ \frac{1}{HT_{\text{opt}} - HT_{\text{min}}} & \text{for } HT_T < HT_{\text{opt}} \end{cases} \]

\[ f_{41}(HT_T) = \begin{cases} 1 & \text{for } HT_T \geq HT_{\text{max}} \\ \frac{1}{HT_{\text{opt}} - HT_{\text{max}}} & \text{for } HT_{\text{opt}} < HT_T < HT_{\text{max}} \\ 0 & \text{for } HT_T < HT_{\text{opt}} \end{cases} \]

In Eq. (10), the coefficient \( p \) is a shape coefficient. When the model was developed, several values for this coefficient were tested successively (deviance information criterion), the final value was set to 0.1.

The coefficients \( HT_{\text{opt}}, a_{w_{\text{opt}}} \), although model parameters, were not estimated but fixed to the following values: \( HT_{\text{opt}} = 85 ^\circ C \), \( T_{\text{opt}} = 37 ^\circ C \), \( a_{w_{\text{opt}}} = 0.999 \). That was done to avoid an over-parameterization in the model equation system. On the other hand, the parameter \( pH_{\text{opt}} \) utilised in the model as a rule (below \( pH_{\text{opt}} \), heat-treatment effect considered, above \( pH_{\text{opt}} \), no heat-treatment effect) was estimated. Overall, the coefficients \( a_1, a_2, T_{\text{min}-1}, T_{\text{min}-2}, pH_{\text{min}-1}, pH_{\text{min}-2}, a_{w_{\text{min}-1}}, HT_{\text{max}} \) and the residual standard deviation \( \sigma_e \) were the other parameters estimated by statistical inference.

2.3. Bayesian inference

To solve the model equation system containing ten estimated parameters a Bayesian approach was chosen for two reasons. Firstly, among the 434 data, 211 were right-censored, meaning that the data recording was stopped before any microbial growth was detected. It appeared natural to express the information provided by the censored data in terms of probability (Eq. (11)) and consequently to use an inference method dealing explicitly with probability distribution functions:

\[ \Pr(\text{Detection time} \geq \text{Time when the experiment was stopped}) \rightarrow 1. \]

Secondly, despite a relatively large amount of data, some additional information was necessary to solve the model equation system. Bayesian technique enables to incorporate previous knowledge on the model parameters into the estimation process, through the settings of prior probability distributions.

The model parameters \( T_{\text{min}-1}, pH_{\text{min}-1}, pH_{\text{min}-2}, pH_{\text{opt}}, a_{w_{\text{min}-1}}, HT_{\text{max}} \) were assumed to follow a Normal distribution, \( N(\text{mean}, \text{variance}) \). Normal distributions for the model parameters are often chosen in Bayesian statistics. The statistic reason for this choice lies in the following mathematical property: with a Normal likelihood and a Normal prior, the posterior distribution is also a Normal distribution (Marin and Robert, 2007). This property is an algebraic convenience; otherwise a difficult numerical integration may be necessary during the model inferring step.

To set the mean of the prior distribution, information provided by either the European Food Safety Agency (2005) or by Experts was used. The variance of the prior was set as follows. Firstly, it was assumed that the temperature varied in a range of a few units, \( pH \) in a range of a few units and tenths, \( a_w \) in a range of a few hundreds and thousandths. Secondly, despite a large amount of data, some additional information was necessary to solve the model equation system. Thus, the values given were rough estimates, to simplify the model implementation, only powers of ten were chosen (\( 10^1, 10^{-1}, 10^{-2} \)).

\[ T_{\text{min}-1} \sim N(4.10) \]

\[ T_{\text{min}-2} \sim N(4.10) \]

\[ pH_{\text{min}-1} \sim N(4.5, 0.1) \]

\[ pH_{\text{min}-2} \sim N(4.5, 0.1) \]

\[ pH_{\text{opt}} \sim N(6.5, 0.1) \]

\[ a_{w_{\text{min}-1}} \sim N(0.92, 0.01) \]

\[ HT_{\text{max}} \sim N(120, 10) \]

To have the coefficient \( a_1 \) and \( a_2 \) positive, they were taken into account in the statistical inference process after the following mathematical transformation:

\[ \ln(a_1) \sim N(0.001, 1000) \]

\[ \ln(a_2) \sim N(0.001, 1000) \]

\[ a_1 = \exp(\ln(a_1)) \text{ and } a_2 = \exp(\ln(a_2)). \]

The standard deviations of the residual error, \( \sigma_e \) was considered unknown, i.e. without particular prior knowledge on its value (Eq. (21)):

\[ \sigma_e \sim \text{Uniform}(0.5, 10). \]

2.4. Model parameter estimation and software

To solve the model equation system and consequently to provide estimates for the model parameters, with their credibility interval, a Markov Chain Monte Carlo algorithm was run. This technique was
carried out with the Winbugs package (version 1.4.3, Medical Research Council, UK). To check the convergence of the iteration process, visual analyses (history function and Gelman and Rubin diagnostic) of three independent chains were performed. A set of 20,000 iterations were run, the first 10,000 iterations were eliminated (burn in period). No convergence problems were detected. The coefficient correlation matrix was deduced from pairwise correlation values, obtained after the discarded 10,000 iterations.

To determine the combinations of process and formulation conditions providing a given detection time (see contour plot in Results section), the set of Eqs. (4)–(10) were solved algebraically.

3. Results

3.1. Model goodness of fit

The model (Eqs. (1) + (3) + (4) + (5) + (7) + (8) + (9) + (10)) was fit to the dataset, using a Bayesian approach. The model outputs provided adjusted values for both strains, and for both censored data and non censored data. Fig. 1 is an attempt to get an overview of the results, however it is imperfect as the censored data (empty symbols) could have been depicted anywhere on the x-axis from the value 4.1 onwards. For short detection time values, ln(detection + 1) corresponding to detection < 3.5 days, the model slightly over-estimated the detection times. Nevertheless, for REPFEDS for which the shelf-life is around 4 weeks, the values below 1 week are of limited interest. Overall, for observed and censored data, the model provided a correct fit, for both strains, the residual model error standard deviation was equal to 1.36.

Before applying the model to design process and formulation conditions suitable for REPFEDs, it was decided to scrutinize the model predictability. That was done with the factors heat-treatment and pH as representative of process and formulation settings. The procedure was set up as follows. From the model, the pH values required to have a detection time of 30 days (same order as REPFED product shelf-life) were calculated for various conditions of heat-treatment temperature and reported in a 2D-plot. This calculation split the experimental domain into two parts: on one hand combination of heat-treatment and pH where growth is observed for Strain 2, censored data

The parameter correlation matrix is given. No high correlation was noticed. The highest values observed were between the coefficients a1 and pHopt (−0.69) and the coefficients a2 and pHopt (−0.67). To verify that the detection time predictions could be derived from the mean values of the parameters, i.e. predictions could be done with the correlations neglected, the following comparison was made. The detection times predicted with the whole probability distribution of each parameter (using Winbugs) were compared to the detection times deduced from the equations in which the parameters were set at their mean values (calculations implemented in Excel). No difference was noticed (data not shown). It was then possible to illustrate the effect of the studied factors on the detection time (sections below) and to calculate the combination of factors delivering a given detection time (last section), using the mean parameter value instead of the whole probability distributions.
3.3. Effect of pH, aw, chilled storage temperature and heat-treatment

The data analysed in this study showed that a combination of stressful conditions significantly extended the detection time. For example, at 8 °C, 13 combinations of heat-treatment, pH and aw were identified that lead to detection times longer than 60 days (Table 6). For these 13 conditions (39 data), the model predictions were in full agreement (no incorrect predictions for either strain). Likewise, at 10 °C, after a pasteurisation at 90 °C for 10 min and with a pH of 5.8, the detection time was predicted to be 11 days in a product at high aw (0.99) but longer than 50 days if the aw dropped to 0.97 (illustration in Fig. 4 with strain 1).

The detection time was predicted to be significantly extended in presence of certain combinations of stressful conditions. Milder conditions, even applied in combination, were not considered sufficient to guarantee a long detection time and then the stability of REPFED. For instance, heat treatment applied at 88 °C for 1 min in a product at pH 5.6, stored at 10 °C, or, a heat treatment slightly stronger (88 °C for 10 min) in a product at pH 5.8 stored at 10 °C (in both case, aw 0.99) did not lead to detection times longer than 10 days (Figs. 4 and 5).

The heat treatment as sole intervention was insufficient to extend the detection time more than a few days. After a heat-treatment at 90 °C for 10 min, required to control non-proteolytic C. botulinum, in a product at pH 6.2, aw 0.99, kept at 10 °C, the detection time was predicted to be no longer than 5 days (Fig. 4). Although extrapolation should be executed with care, the model did not predict a much longer detection time when a stronger heat treatment was applied (either by longer heat-treatment time or heat-treatment temperature) if the storage temperature remained at 10 °C, and pH and aw conditions were not stressful. For instance, with a heat-treatment of 95 °C for 10 min, the

![Fig. 2. pH versus heat-treatment temperatures, applied for 33 min, at 10 °C, for aw of 0.973 (A), 0.98 (B), 0.987 (C) and 0.995 (D). Observed values were deliberately spread around their values to get all them visible: detection time < 30 days (x) and censored values with detection time > 60 days (o). When the observed detection time was close to 30 days, values were reported with a different symbol (▲) and labelled. Predicted detection time at 30 days for strain 1 (solid line) and strain 2 (dotted line).](image)

### Table 3
Comparison between model predictions and experimental results obtained with non-heated spores (data not used for building the predictive time-to-detect growth model).

<table>
<thead>
<tr>
<th>Storage temperature (°C)</th>
<th>pH</th>
<th>aw</th>
<th>Number of experiments</th>
<th>Results</th>
<th>Model predictions (based on strain 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>5.2</td>
<td>0.973</td>
<td>2</td>
<td>&gt;60 days</td>
<td>&gt;60 days</td>
</tr>
<tr>
<td>8</td>
<td>5.2</td>
<td>0.98</td>
<td>2</td>
<td>&gt;60 days</td>
<td>&gt;60 days</td>
</tr>
<tr>
<td>8</td>
<td>5.6</td>
<td>0.973</td>
<td>2</td>
<td>&gt;60 days</td>
<td>&gt;60 days</td>
</tr>
<tr>
<td>8</td>
<td>5.6</td>
<td>0.98</td>
<td>2</td>
<td>&gt;60 days</td>
<td>35 days</td>
</tr>
<tr>
<td>10</td>
<td>5.2</td>
<td>0.973</td>
<td>4</td>
<td>&gt;60 days</td>
<td>&gt;60 days</td>
</tr>
<tr>
<td>10</td>
<td>5.2</td>
<td>0.98</td>
<td>4</td>
<td>&gt;60 days</td>
<td>&gt;60 days</td>
</tr>
<tr>
<td>10</td>
<td>5.6</td>
<td>0.973</td>
<td>4</td>
<td>&gt;60 days</td>
<td>20 days</td>
</tr>
<tr>
<td>10</td>
<td>5.6</td>
<td>0.98</td>
<td>4</td>
<td>[15.9–55.8]</td>
<td>12 days</td>
</tr>
</tbody>
</table>

* Range of observed lag times (days).

### Table 4
Estimated model parameters: mean, median and 95% credibility interval.

<table>
<thead>
<tr>
<th>Model parameter</th>
<th>Mean</th>
<th>sd</th>
<th>2.50%</th>
<th>Median</th>
<th>97.50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a_1$</td>
<td>0.68</td>
<td>0.12</td>
<td>0.44</td>
<td>0.68</td>
<td>0.90</td>
</tr>
<tr>
<td>$a_2$</td>
<td>1.11</td>
<td>0.20</td>
<td>0.72</td>
<td>1.11</td>
<td>1.52</td>
</tr>
<tr>
<td>HTmax</td>
<td>120.3</td>
<td>3.1</td>
<td>114.2</td>
<td>120.3</td>
<td>126.4</td>
</tr>
<tr>
<td>$T_{\text{min-1}}$</td>
<td>4.94</td>
<td>0.84</td>
<td>3.01</td>
<td>5.05</td>
<td>6.26</td>
</tr>
<tr>
<td>$T_{\text{min-2}}$</td>
<td>3.36</td>
<td>1.69</td>
<td>−0.23</td>
<td>3.42</td>
<td>6.43</td>
</tr>
<tr>
<td>$a_{\text{min-1}}$</td>
<td>0.923</td>
<td>0.016</td>
<td>0.881</td>
<td>0.927</td>
<td>0.944</td>
</tr>
<tr>
<td>pH_{min-1}</td>
<td>4.48</td>
<td>0.11</td>
<td>4.25</td>
<td>4.49</td>
<td>4.65</td>
</tr>
<tr>
<td>pH_{min-2}</td>
<td>4.17</td>
<td>0.14</td>
<td>3.83</td>
<td>4.19</td>
<td>4.40</td>
</tr>
<tr>
<td>pH_{opt}</td>
<td>6.23</td>
<td>0.25</td>
<td>5.94</td>
<td>6.16</td>
<td>6.99</td>
</tr>
<tr>
<td>$\sigma_\varepsilon$</td>
<td>1.36</td>
<td>0.07</td>
<td>1.23</td>
<td>1.35</td>
<td>1.50</td>
</tr>
</tbody>
</table>
predicted detection time was still lower than one week when pH equalled 6.2 (Fig. 4). Likewise, the pH on its own was not predicted to inhibit the spore germination and outgrowth process for long. Even at pH 5.6, if the heat treatment is mild (88 °C for 1 min), the detection time was predicted to be no longer than eight days (Fig. 5).

The equivalence in pasteurisation settings was analysed. With a z-value of 9.6 °C (Daelman et al., 2013), 90 °C for 10 min is equivalent to 87 °C for 21 min, and also to 95 °C for 3 min. These three pasteurisation regimes were assessed to be equivalent in terms of detection time: respectively 13, 12 and 11 days in product at pH 6.0, aw 0.98, stored at 10 °C.

In conclusion, only the combination of chilled temperature (10 °C or lower), acidic pH conditions (pH 5.8 or lower), sub-optimal aw conditions (aw 0.98 or lower) and moderate thermal treatment (e.g. 90 °C for 10 min or higher) enabled to extend the detection time significantly (e.g. more than three weeks) and consequently to assure product stability of REPPEF. More combinations of process and formulation conditions are provided in the next section.

3.4. Combinations of process and formulation conditions able to deliver a given detection time

Combinations of formulation and storage temperature delivering a given detection time can be generated directly from the equation system. In Figs. 6 and 7, examples of contour plots are presented for detection times of 30 days or 50 days (with strain 1). As indicated in Fig. 6, with a mild heat-treatment (90 °C for 1 min), the detection time will be shorter than 30 days if the storage temperature is higher than 10 °C unless the pH is acidic (e.g. pH 5.3 at 10 °C, or pH 5.0 at 18 °C). This result means that during REPPEF preparation, for example after mixing and first cooking of ingredients, the plant room temperature is too high to expect any significant detection time and thus significant spore lag time.

In Fig. 7, various conditions of pH and aw that enable an extension of the detection time up to 30 days, after the commonly applied heat treatment of 90 °C for 10 min, are presented. During storage of products, at chilled conditions, even if the average temperature is around 6 °C, there is a risk to have temperature as high as 10 °C (Derens et al., 2004). At this storage temperature and with heat treatment using the standard thermal value of 90 °C for 10 min, pH and aw have to act in combination to extend the detection time to 30 days: pH 6.0–aw 0.97, pH 5.7–aw 0.98 or pH 5.5–aw 0.99 are conditions suitable to guarantee the desired detection time (Fig. 7).

4. Discussion

A mathematical model quantitatively assessing the effect of heat treatment, pH, aw and storage temperature on the detection time of B. cereus heat-treated spores was developed. To our best knowledge, there is no such model with either detection time or lag time as response, publicly available in either literature or databases. Gaillard et al. (2005) developed a heat-treated B. cereus spore lag time, but taking into account only heat treatment and pH (experiments performed at a storage temperature of 25 °C, without any variation of aw). Modeling thermally stressed spore lag time is difficult as the biological mechanism involved is complex (Augustin, 2011), it encompasses spore damage, spore repair and cell outgrowth (Smelt et al., 2008; Ter Beek et al., 2011) and definitively more difficult to quantify than a vegetative cell lag time.

In the model presented here, the residual error corresponded mainly to uncertainty, first of all due to the lack of replicates in the experimental design, i.e. with a higher number of replicates it would have been smaller. This uncertainty has various sources. In our case, the statistical response analysed was not exactly the lag time before

Table 5
Model parameter correlation matrix.

<table>
<thead>
<tr>
<th>Model parameter</th>
<th>a1</th>
<th>a2</th>
<th>HTmax</th>
<th>Tmin-1</th>
<th>Tmin-2</th>
<th>Tmin-1</th>
<th>Tmin-1</th>
<th>pHmin-1</th>
<th>pHmin-2</th>
<th>pHopt</th>
</tr>
</thead>
<tbody>
<tr>
<td>a1</td>
<td>1</td>
<td>0.56</td>
<td>0.06</td>
<td>-0.39</td>
<td>-0.02</td>
<td>-0.24</td>
<td>-0.39</td>
<td>-0.06</td>
<td>-0.06</td>
<td>-0.69</td>
</tr>
<tr>
<td>a2</td>
<td>1</td>
<td>0.06</td>
<td>0.12</td>
<td>0.003</td>
<td>0.01</td>
<td>0.13</td>
<td>0.13</td>
<td>0.01</td>
<td>0.01</td>
<td>-0.01</td>
</tr>
<tr>
<td>HTmax</td>
<td>1</td>
<td>1</td>
<td>-0.03</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Tmin-1</td>
<td>1</td>
<td>0.01</td>
<td>0.11</td>
<td>0.03</td>
<td>0.37</td>
<td>0.05</td>
<td>0.04</td>
<td>0.34</td>
<td>0.34</td>
<td>0.34</td>
</tr>
<tr>
<td>Tmin-2</td>
<td>1</td>
<td>1</td>
<td>0.004</td>
<td>0.05</td>
<td>0.03</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Table 6
Data collected at 8 °C at various conditions of heat-treatment temperature (HTT) and time (HTt), pH and aw. All these data were censored values (detection time > 60 days) whatever the strain.

<table>
<thead>
<tr>
<th>HTT (°C)</th>
<th>HTt range (min)</th>
<th>pH</th>
<th>aw</th>
<th>Number of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>85</td>
<td>27–38</td>
<td>5.2</td>
<td>0.973</td>
<td>5</td>
</tr>
<tr>
<td>87</td>
<td>12–25</td>
<td>5.2</td>
<td>0.973</td>
<td>6</td>
</tr>
<tr>
<td>85</td>
<td>27</td>
<td>5.2</td>
<td>0.980</td>
<td>1</td>
</tr>
<tr>
<td>85</td>
<td>33–38</td>
<td>5.2</td>
<td>0.987</td>
<td>3</td>
</tr>
<tr>
<td>85</td>
<td>12–25</td>
<td>5.2</td>
<td>0.987</td>
<td>5</td>
</tr>
<tr>
<td>85</td>
<td>27–33</td>
<td>5.2</td>
<td>0.995</td>
<td>3</td>
</tr>
<tr>
<td>87</td>
<td>12</td>
<td>5.2</td>
<td>0.995</td>
<td>2</td>
</tr>
<tr>
<td>87</td>
<td>20–25</td>
<td>5.6</td>
<td>0.973</td>
<td>2</td>
</tr>
<tr>
<td>85</td>
<td>33–38</td>
<td>5.6</td>
<td>0.980</td>
<td>4</td>
</tr>
<tr>
<td>85</td>
<td>27</td>
<td>5.6</td>
<td>0.987</td>
<td>2</td>
</tr>
<tr>
<td>87</td>
<td>20–25</td>
<td>5.6</td>
<td>0.987</td>
<td>3</td>
</tr>
<tr>
<td>87</td>
<td>12–20</td>
<td>5.6</td>
<td>0.995</td>
<td>3</td>
</tr>
</tbody>
</table>

Fig. 3. Predicted detection times versus heat-treatment temperatures for various pH conditions and for the two strains. Heat-treatment time of 10 min, storage temperature of 10 °C, aw 0.98.
germination but the sum of two times: time required to have spore germination and outgrowth, and time to have the subsequent vegetative cells growing up to a detectable level. In addition, the detection time could vary as function of the experimental design (the wells were not systematically checked every day). The uncertainty was also due to the large set of censored data, imprecise by definition. Besides uncertainty linked to the data, there was also a non negligible uncertainty due to the model. It might be explained by the model constraints: based on monotonous and algebraically simple functions. A linear model with a polynomial function of degree three or more might have provided a better fit. That was not the choice made here, for reason of practicality in future use, i.e. for designing process and formulation delivering a given detection time. In addition, the model uncertainty might be due to the use of existing data, not generated in the first place to develop a time-to-detect growth model, but a growth/no-growth interface model. In the latter case, the experimental design is set up around the interface and then automatically many censored data are generated (no-growth interface). Nevertheless, developing predictive models using existing data is still a valuable desk-exercise to attempt as it is less costly than generating data from scratch.

On the other hand, the phenomenon undergone by heat-treated B. cereus spores before germinating and recovering in stressful conditions has been reported as complex and naturally variable (Hornstra et al., 2009). Likewise, Stringer et al. (2011) studying the lag time of C. botulinum spores at a single cell level, emphasized that the variability in individual spore responses was high. This spore response variability comes on the top of uncertainty and is also included in the residual error. The model developed in our study includes the two strains within a single structure, the residual error was considered to be the same whatever the strain. Such hierarchical non linear model is easy to set up in a Bayesian framework. Another advantage of a Bayesian approach is the possibility of combining Experts’ opinion and data in the model parameter estimation process. In our study, Experts were asked to set the model parameters either to a single likely value (HTopt, T opt and awopt) or to an informative probability distribution (Tmin-1, Tmin-2, pHmin-1, pHmin-2, pHopt, awoptmin-1 and HTmax). More generally, Bayesian inference has already been used successfully in food microbiology, for instance to build hierarchical model (Busschaert et al., 2011; Crépet et al., 2009; Membré et al., 2011) or growth models (Jaloustre et al., 2011; Pouillot et al., 2003).

The effect of heat-treatment on B. cereus spore injury has been already reported (Faille et al., 1997). Likewise, an increased inhibitory effect (longer lag time) on germination of B. subtilis spores after heating.
at 90 °C for 5 min compared to spores that were not heated, has been established (Ciarciaglini et al., 2000). With non-proteolytic C. botulinum spores, it has been reported that due to thermal injury, the time for the spores to recover is prolonged (Peck et al., 1995). Interestingly, in the studies mentioned above, the combination of effects due to heat-treatment and pH is not mentioned while in our study, the heat treatment as sole intervention measure was not sufficient to guarantee stability of products. On the other hand, with C. botulinum, Stringer et al. (2011) came to the same conclusions as ours, the authors emphasized that the spore lag time depended on both the historic treatment of the spores and the prevailing growth conditions.

Contour plots are useful graphical tools to visualize the combinations of process and formulations delivering a given detection time; as such, contour plots can be assimilated to decision tools for R&D product designers and risk assessors. In this study, contour plots were easy to generate since the mathematical expressions chosen to define the model were monotonous and had algebraic solutions. These mathematical constraints were added deliberately in the model development to obtain an easy-to-use predictive model helping in food product design. The drawback of this choice was that the constraints could penalize the model accuracy. A Gamma multiplicative structure, although modified, seems appropriate for such model development. Gamma models have already a long history of successful applications in the food safety domain as they have been extensively used for describing the effect of temperature, pH and $a_w$ and preservative agents on the growth rates. In such a case, a square root transformation of the growth rate is often performed to stabilise the variance (Ross and Dalgaard, 2004), meaning that the multiplicative Gamma structure is maintained despite the transformation: $Y = Y + e, \text{ with } Y = f(T) \times g(\text{pH}) \times h(\text{aw}) \times ...$. Such multiplicative Gamma structure has highly valuable advantages over other model structures: parsimony in parameters, facility of interpretation of the relative impact of each inhibitory factor, and flexibility in adding new inhibitory factors to the model.

There are not many applications of the Gamma structure on lag times. So far, in predictive microbiology, the lag phase has often been interpreted as a period during which a certain amount of work has to be done to enable subsequent growth (Baranyi and Pin, 2004; Robinson et al., 1998). The work-to-be-done can be written as the multiplication of the growth rate and the lag time (Koutsoumanis, 2001). It has often been assumed that the work-to-be-done is a constant whatever the environmental conditions (Ross and Dalgaard, 2004) and then, only the growth rate has been modelled (the lag time could be derived from the growth rate model). Here, the statistical response was “In (detection + 1)”, close to “ln(lag + 1)” as the detection time was for a large part explained by the lag phase (the inoculums was high). The logarithm transformation of the lag time is rather frequent in food microbiology. For instance, in one of the first key papers in predictive microbiology, Zwietering et al. (1994) evaluated various data transformation and concluded that the logarithm transformation for lag times values was recommended. From a large set of data collected on L. monocytogenes, Augustin and Carlier (2000) predicted the lag time after a log transformation. Working with B. cereus, in an attempt to predict the lag time after a heat treatment, Gaillard et al. (2005) suggested a simple model, based on a logarithm transformation. Likewise, Smelt et al. (2002) working on Lactobacillus plantarum cells injured by a heat treatment, modelled the lag time distribution after a logarithm transformation.

The structure of the time-to-detect growth model applied to heat-treated spore, developed here is close to a multiplicative one: $Y = Y + e$ and $Y = f(T) \times g(\text{pH}) \times h(\text{aw}) \times ...$. The results are satisfactory enough to consider that despite the log transformation, the Gamma multiplicative structure was still relevant. However, the extra parameter “−1” may lead to negative detection time when the factors storage temperature, pH, $a_w$, and heat treatment are all together near their optimal values. Development of heat-treated spore lag time models should increase in the near future.

In conclusion, a mathematical model predicting the time-to-detect growth of B. cereus heat-treated spore has been developed. The heat treatment was applied after spore inoculation to build a model which can be included in a quantitative risk assessment model mimicking a production process from raw materials to product consumption. Indeed, the spores are likely to be present in the raw material and then mixed with the product preparation, at a given $a_w$ and pH, before packaging and pasteurization.

The model describes the effect of thermal process and product formulation ($\text{pH and } a_w$) on B. cereus behaviour, at chilled conditions. When building the model, we deliberately chose to inoculate the two strains at a high level to obtain a detection time relatively close to the lag time. When applying the time-to-detect growth model to a REPFED in order to determine the end-product pack contamination level, it will be necessary to combine the post-process contamination level, the detection time and the growth rate, to calculate the level in the end-product. In REPFEDs, the post-process contamination level is likely to be low (Carlin et al., 2000; Del Torre et al., 2001), and knowing that a low spore concentration decreases the probability of germation (Abee et al., 2011; Caipo et al., 2002; Peck and Stringer, 2005), the model developed in our study might slightly over-estimate the actual lag time in REPFEDs.

Once the model validated, it might be incorporated into a quantitative risk assessment model to assess the risk related to B. cereus in REPFEDs, or use on its own to suggest combinations of process and formulation delivering a given detection time.

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