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

Jeff Daelman [a], Aditya Sharma [b,c], An Vermeulen [a,d], Mieke Uyttendaele [a], Frank Devlieghere [a,d], Jeanne-Marie Membré [b,c,*]

[a] FMFP, Laboratory of Food Microbiology and Food Preservation, Department of Food Safety and Food Quality, Ghent University, Coupure Links 653, 9000 Ghent, Belgium [1]
[b] INRA, UMR1014 SECALIM, Rue de la Géraudière, CS 82225, 44322, Nantes Cedex 3, France
[c] LUNAM Université, ONIRIS, Nantes, France
[d] CPFM2, Flemish Cluster Predictive Microbiology in Foods, Department of Food Safety and Food Quality, Ghent University, Coupure Links 653, 9000 Ghent, Belgium

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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 (a_w), heat treatment and storage temperature was developed. As the inoculum was high, the time-to-detect growth was the sum of two times: for a large part of the spore lag time (time before germination and outgrowth) and to a lesser extent of the time to have subsequent vegetative cells growing up to a detectable level.

A dataset of 434 combinations (of pH, a_w, heat treatment, storage temperature and *B. cereus* strain), originally collected at Ghent University to build a growth/no-growth model for two *Bacillus cereus* strains, was re-interpreted as time-to-detect growth values. In the growth area (223 combinations) 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 a_w (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 a_w 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 a_w such as pH 6.0–a_w 0.97, pH 5.7–a_w 0.98 or pH 5.5–a_w 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 REPFEDs is generally in a range of two/three up to five/six weeks depending on the production process and product formulation. In REPFEDs, 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

* Corresponding author at: INRA, UMR1014 SECALIM, Rue de la Géraudière, CS 82225, 44322, Nantes Cedex 3, France. Tel./fax: +33 25178 5521.
E-mail address: jeanne-marie.membre@oniris-nantes.fr (J.-M. Membré).
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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; Martinez 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 °C), the lag time values were relatively short even under stressful conditions, for instance, at temperature 7 °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 outgrowth, 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 $1.3\,10^6$ CFU/ml, and consequently the spore inoculum was deliberately high (10^4–10^5) 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 $D_{90\,°C}$-value of 90.9 min, while spores of strain 2 have a $D_{90\,°C}$-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 10^4–10^5 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.

$$\ln(\text{lag}+1) = a_s \times \prod_i^k \gamma_{is} - 1 + \varepsilon \quad (1)$$

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 γ_i terms corresponding to the four factors ($k = 4$): storage temperature (StT), pH, a_w and heat treatment (HT, 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, ε, was assumed to be normally distributed: $\varepsilon \sim N(0, \sigma_\varepsilon^2)$. The left hand side of Eq. (1) was written as $\ln(\text{lag} + 1)$ 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 StT, 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), $\ln(\text{lag} + 1) = 0$ is equivalent to $\text{lag} = 0$) when the factors were not at their optimal values and meant that the coefficient a_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)):

$$\text{lag} + 1 = \exp\left(a_s \times \prod_i^k \gamma_{is}\right) / \exp(1). \quad (2)$$