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Modeling heat resistance of *Bacillus weihenstephanensis* and *Bacillus licheniformis* spores as function of sporulation temperature and pH

Running title: Modeling resistance of *Bacillus* spores formed in various environments

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Abstract

Although sporulation environmental factors are known to impact on *Bacillus* spore heat resistance, they are not integrated into predictive models used to calculate the efficiency of heating processes. This work reports the influence of temperature and pH encountered during sporulation on heat resistance of *B. weihenstephanensis* KBAB4 and *B. licheniformis* AD978 spores. A decrease in heat resistance (δ) was observed for spores produced either at low temperature, at high temperature or at acidic pH. Sporulation temperature and pH maximizing the spore heat resistance were identified. Heat sensitivity (z) was not modified whatever the sporulation environmental factors were. A resistance secondary model inspired by the Rosso model was proposed. Sporulation temperatures and pHs minimizing or maximizing the spore heat resistance ($T_{min(R)}$, $T_{opt(R)}$, $T_{max(R)}$, $pH_{min(R)}$ and $pH_{opt(R)}$) were estimated. The goodness of the model fit was assessed for both studied strains and literature data. The estimation of the sporulation temperature and pH maximizing the spore heat resistance is of great interest to produce spores assessing the spore inactivation in the heating processes applied by the food industry.

Key words: Predictive microbiology, Model, Heat resistance, Sporulation environment, *Bacillus weihenstephanensis*, *Bacillus licheniformis*

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1 Introduction

Bacillus spp. are widely found in food and industrial environment (Anonymous, 2005). Because of their implication in food poisoning and food spoilage, *Bacillus* spp. inactivation is a major objective in food processing (De Jonghe *et al.*, 2010; Nauta *et al.*, 2003; Scheldeman *et al.*, 2005). As *Bacillus* spores are able to resist to harsh environmental conditions, the calculation of the process efficiency is necessary to ensure food safety. The variation of spore heat resistance as a function of the sporulation environment has been extensively reported. The increase in sporulation temperature leads to an increase in spore heat resistance of several *Bacillus* species (Baweja *et al.*, 2008; Garcia *et al.*, 2010; Gonzalez *et al.*, 1999; Palop *et al.*, 1999; Planchon *et al.*, 2011). At high sporulation temperatures spore resistance reaches a plateau or decreases (Gonzalez *et al.*, 1999; Lindsay *et al.*, 1990). A Bigelow-like log linear model was proposed to fit the increase of spore heat resistance; unfortunately this was not able to describe such a plateau (Leguerinel *et al.*, 2007). The increase in pH 6.0 to 8.1 in buffered sporulation media also caused a significant increase in *B. cereus* spore heat resistance (Mazas *et al.*, 1997). No significant difference of heat resistance of spores formed in non-buffered media at various initial pHs was reported (Craven, 1990; Mazas *et al.*, 1997). Sporulation environment induces obvious variations of spore heat resistance, but is not taken into account in heat process calculations (Carlin, 2011). The aim of this study was to propose a model describing the spore heat resistance as a function of the sporulation environment. To test the robustness of the model, the influence of sporulation temperature and pH was studied on the spore heat resistance of the psychrotrophic species *Bacillus weihenstephanensis* and of the mesophilic species *Bacillus licheniformis*.

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2 Materials and methods**2.1 Bacterial strains and cell preparation**

Bacillus weihenstephanensis KBAB4 strain isolated from a forest soil (Vilas-Boas *et al.*, 2002) was kindly provided by the Institut National de la Recherche Agronomique (INRA, Avignon, France). *B. weihenstephanensis* KBAB4 strain belongs to the psychrotrophic group VI of *B. cereus sensu lato* (Guinebrethiere *et al.*, 2008). *Bacillus licheniformis* AD978 strain, isolated from raw dairy ingredients, was kindly provided by ADRIA Développement (Quimper, France).

The strains were frozen (-20°C) as 1 mL aliquots in nutrient broth diluted in 50% glycerol (v/v). A 1 mL aliquot was inoculated in 100 mL nutrient broth (Biokar Diagnostics, Beauvais, France) contained in 250 mL flasks and incubated for 8 h. All cultures were shaken at 100 rpm, incubated at 30°C for the *B. weihenstephanensis* KBAB4 strain and at 45°C for the *B. licheniformis* AD978 strain. A 1 mL sample of these cultures was diluted in 100 mL broth and was overnight incubated. A 0.1 mL sample of the overnight culture of *B. weihenstephanensis* KBAB4 or 0.01 mL sample of the overnight culture of *B. licheniformis* AD978 was diluted again in 100 mL nutrient broth and incubated for 6 h. The final cell concentrations were approximately of 5.10^7 CFU/mL with less than 200 spores CFU/mL (resistant cells to a heat treatment at 70°C for 5 min) for both strains. These last cultures were used for spore production.

2.2 Spore production

Vegetative cells obtained from cell preparation were inoculated in a sporulation mineral buffer (SMB) (Baril *et al.*, 2011) or in modified nutrient agar (MNA) to obtain spores. Whatever the sporulation conditions, sporulation efficiency evaluated by the final spore

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concentration and the sporulation duration was in accordance with literature results (Baril *et al.*, submitted paper).

The SMB was composed of phosphate buffer (K_2HPO_4/KH_2PO_4 , 39 mM) supplemented with $CaCl_2 \cdot 2H_2O$ (8.0 mg/L) and $MnSO_4 \cdot H_2O$ (1.5 mg/L) and sterilized by filtration on a 0.22 μm filter (Steritop system; Millipore Corporation, Billerica, MA). The sporulation pH ranging from pH 5.5 to 8.5 was adjusted by modifying the composition of the phosphate buffer in SMB and was checked after sterilization. To inoculate the SMB, the 100 mL cell suspension was centrifuged (6000 x g, 10 min, 12°C). The pellet was resuspended in 100 mL SMB and was then incubated at the desired temperature from 5°C to 50°C under shaking at 100 rpm. When more than 95% of free spores were observed under a phase contrast microscope, the SMB was centrifuged (6000 x g, 10 min, 12°C). The spore pellet was suspended in 5 mL distilled water and stored for one month at 4°C before experimental use.

Modified nutrient agar (MNA), composed of nutrient agar (Biokar diagnostics, Beauvais, France) supplemented with $CaCl_2 \cdot 2H_2O$ (100 mg/L) and $MnSO_4 \cdot H_2O$ (50 mg/L) was sterilized by autoclaving at 120°C for 20 min. The MNA plates were inoculated with 0.5 mL of the cell culture and were incubated at temperature of 10°C, 30°C and 35°C for *B. weihenstephanensis* KBAB4 and of 20°C, 45°C and 50°C for *B. licheniformis* AD978.

The sporulation time was determined as in SMB by the time to obtain at least 95% free spores. MNA surfaces were then scrapped to suspend spores in distilled water. Spore suspension was centrifuged (6000 x g, 10 min, 12°C) and pellet was resuspended in 5 mL distilled water and stored for one month at 4°C before experimental use.

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2.3 Determination of spore heat resistance

Capillary tubes of 200 μ L (Hirschman Laborgerate, Eberstadt, Germany) were filled with 100 μ L of spore suspension and sealed (Baril *et al.*, 2011). Heat treatments were carried out in thermo-stated glycerol/water bath at 80°C, 85°C, 90°C, 95°C and 100°C for *B. weihenstephanensis* KBAB4 and at 85°C, 90°C, 95°C, 100°C and 105°C for *B. licheniformis* AD978. At each target heating time, one capillary tube was cooled in a water-ice bath and washed with ethanol. Extremities were blazed and broken. Decimal dilutions of the capillary tube content mixed to 0.9 mL of a rinsing solution made of tryptone salt broth (Biokar Diagnostics, Beauvais, France) were spread on nutrient agar plates using a spiral plater (WASP1; Don Whitley, Shipley, West Yorkshire, United Kingdom). Plates of *B. weihenstephanensis* KBAB4 strain were incubated at 30°C and those of *B. licheniformis* AD978 strain at 45°C for 24 h before colony enumeration. Cell concentrations were calculated and expressed as colony forming units per mL (CFU/mL). The initial spore concentration of each survival curves was determined by heating one capillary tube at 70°C for 5 min.

2.4 Experimental design

The influence of the sporulation environment, temperature and pH, was studied on spore heat resistance of two bacillus species, *B. weihenstephanensis* and *B. licheniformis*. To assess the independence between the evolution of spore heat resistance and the sporulation medium, two sporulation media, SMB and MNA, were tested. The whole data sets were acquired from univariate experiments. When the sporulation temperature was studied the sporulation pH was fixed at 7.2 and when the sporulation pH was studied the sporulation temperature was fixed at 30°C for *B. weihenstephanensis* and at 45°C for *B. licheniformis*.

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The influence of sporulation temperature was studied in SMB in the range of 5°C to 35°C for *B. weihenstephanensis* and of 20°C to 50°C for *B. licheniformis*. Spores were also produced in MNA at 10°C, 30°C and 35°C for *B. weihenstephanensis* and at 20°C, 45°C and 50°C for *B. licheniformis*. At least three replicates were performed for each sporulation media, as independent spore production, at 10°C, 30°C and 35°C for *B. weihenstephanensis*, and at 20°C, 45°C and 50°C for *B. licheniformis*.

The influence of sporulation pH was only studied in SMB in the range of pH 5.5 to 8.5 for *B. weihenstephanensis* and of pH 6.3 to 8.5 for *B. licheniformis*. At least three replicates were performed at pH 5.9 and 7.2 for *B. weihenstephanensis* and at pH 6.3 and 7.2 for *B. licheniformis*.

2.5 Models

2.5.1 Resistance primary model

The Weibull model modified by Mafart *et al.* (2002) was used to fit survival curves (Equation 1):

$$\log(N) = \log(N_0) - \left(\frac{t}{\delta}\right)^p \quad (1)$$

where N (CFU/ml) is the population concentration at time t , N_0 (CFU/ml) is the initial population concentration, δ (min) is the time to the first decimal reduction at a given heating temperature and p is a shape parameter.

2.5.2 Resistance secondary model

The gamma-concept (Zwietering *et al.*, 1992) and the cardinal growth model (Rosso *et al.*, 1995) were associated to fit the influence of sporulation temperature and pH on spore heat resistance (Equation 2). The resistance secondary model was composed of two

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independent gamma modules, one for the sporulation temperature ($\gamma_2(T_{spo})$) and another for the sporulation pH ($\gamma_1(pH_{spo})$).

$$\delta = \delta_{\max} \cdot \gamma_2(T_{spo}) \cdot \gamma_1(pH_{spo}) \quad (2)$$

where δ is the time to the first decimal reduction of spores produced at a given sporulation temperature T_{spo} and a given sporulation pH_{spo} . $\delta_{\max}$ is the value of δ predicted at sporulation temperature and pH allowing the maximal resistance.

Gamma is comprised between 0, at non-optimal conditions, and 1, at optimal conditions. When sporulation temperature and pH are optimal, the spore heat resistance is maximal, meaning equal to $\delta_{\max}$. The gamma modules of sporulation temperature and pH were adapted from the cardinal growth model (Rosso *et al.*, 1995), as the following equation (3):

$$\gamma_n(X_{spo}) = \begin{cases} 0.0 & X \leq X_{\min(R)} \\ \frac{(X - X_{\max(R)})(X - X_{\min(R)})^n}{(X_{opt(R)} - X_{\min(R)})^{n-1} [(X_{opt(R)} - X_{\min(R)})(X - X_{opt(R)}) - (X_{opt(R)} - X_{\max(R)})(n-1)X_{opt(R)} + X_{\min(R)} - nX]} & X_{\min(R)} < X < X_{\max(R)} \\ 0.0 & X \geq X_{\max(R)} \end{cases} \quad (3)$$

$X_{\min(R)}$ and $X_{\max(R)}$ are the theoretical minimal and maximal sporulation temperature or pH between which the spore heat resistance is higher than zero. $X_{opt(R)}$ is the sporulation temperature or pH where spores resistance is maximal. The investigated pH range did not allow estimating the $pH_{\max(R)}$ parameter. As for the cardinal growth model (Membre *et al.*, 2002), $pH_{\max(R)}$ was assumed to be symmetrical to $pH_{\min(R)}$ with respect to $pH_{opt(R)}$ and was replaced by $2pH_{opt(R)} - pH_{\min(R)}$. Thus, at $T_{opt(R)}$ and at $pH_{opt(R)}$, spore heat resistance (δ value) is maximal and equal to $\delta_{\max}$. At $T_{\min(R)}$, $T_{\max(R)}$ and $pH_{\min(R)}$, as mathematical consequence, spore heat resistance (δ value) is equal to zero.

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The influence of the heating temperature on spore heat resistance was estimated by the Bigelow model (Mafart *et al.*, 2002) (Equation 4):

$$\delta = \delta^* \cdot 10^{-\left(\frac{T_{HT} - T_{HT}^*}{z}\right)} \quad (4)$$

where δ^* is the δ value for a treatment at a reference heating temperature (T_{HT}^*). T_{HT} is the heating temperature. z (°C), the heat sensitivity, corresponds to the increase of the heating temperature causing a decimal reduction of δ values.

In order to describe both the influence of the sporulation environmental factors and of heating temperature on spore heat resistance, equation (2) was combined in equation (4) as follows:

$$\delta = \delta_{\max}^* \gamma_2(T_{spo}) \gamma_1(pH_{spo}) 10^{-\left(\frac{T_{HT} - T_{HT}^*}{z}\right)} \quad (5)$$

where $\delta_{\max}^*$ is the δ value acquired by spores produced at the optimal sporulation temperature ($T_{opt(R)}$) and pH ($pH_{opt(R)}$) for a treatment at the reference heating temperature (T_{HT}^*).

2.5.3 Model fit and statistical analysis

In order to reduce the variance of the studied responses (N and δ), the model parameters were estimated by minimizing the sum of squared error (SSE) of the decimal logarithm of the population size, meaning $\log(N)$ values for the resistance primary model (Equation 1) and the decimal logarithm of the heat resistance, meaning $\log(\delta)$ values for the resistance secondary model (Equation 5). These estimations were computed from experimental data sets by non linear regression (lsqcurvefit function, Optimization Toolbox; MATLAB 7.9.0; The Math-works), as well as the estimation of the 95% confidence intervals (nlparci, Statistical Toolbox; MATLAB 7.9.0; The Math-works). The goodness of fit was

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assessed by the root mean square error (RMSE). The smaller the RMSE was the best the model fit was. In addition, to test the robustness of the resistance secondary model, the literature available data set of *B. cereus* ATCC7004 (Gonzalez *et al.*, 1999) was fitted from the resistance secondary model.

The likelihood ratio test was used to test whether a unique shape parameter p or unique environmental resistance parameters ($T_{min(R)}$, $T_{opt(R)}$, $T_{max(R)}$, $pH_{min(R)}$ and $pH_{opt(R)}$) could be estimated. Thus from the primary model, one N_0 , one δ and one p values were estimated for each survival curve; in a second fitting procedure one N_0 and one δ were estimated for each survival curve but a unique shape parameter p was estimated for all the survival curves in one fitting procedure (Couvert *et al.*, 2005; Fernández *et al.*, 2002; Jagannath *et al.*, 2005). In the same way, from the secondary model, each environmental parameters were estimated for each heating temperature (one δ , one $T_{min(R)}$, one $T_{opt(R)}$, one $T_{max(R)}$, one $pH_{min(R)}$ and one $pH_{opt(R)}$ values); in a second fitting procedure, one δ was estimated for each heating temperature but unique $T_{min(R)}$, $T_{opt(R)}$, $T_{max(R)}$, $pH_{min(R)}$ and $pH_{opt(R)}$ values were overall estimated for all the heating temperature in one fitting procedure. The likelihood ratio test compares the goodness of fit of two models: the model without constrain and the unique estimated parameter model. The unique estimated parameter model fit consists in estimating the parameters under the constraint of the same value of each parameter whatever the data set. The null hypothesis corresponded to no significant differences between the goodness of fit of both models. The likelihood ratio test (S_L) was calculated as follow (Huet *et al.*, 2003):

$$S_L = n \cdot \ln \left(\frac{SSE_u}{SSE_w} \right) \quad (6)$$

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Where n is the number of experimental data, SSE_w is the sum of squared errors obtained from the model fit without constraint and SSE_u is the sum of squared errors obtained from the under-constraint model fit. The freedom degree was defined as the difference between the parameter number estimated by the model without constraint and by the under-constraint model. The null hypothesis was rejected when the S_L value was higher than the Chi-squared distribution for $\alpha=5\%$.

As variances of spore heat resistance ($\log(\delta)$) were heterogeneous, the Kruskal-Wallis test was computed to test the influence of sporulation environment on the spore heat resistance (kruskalwallis and ranksum functions, Statistics Toolbox; MATLAB 7.9.0; The Math-works). The covariance analysis was performed to study the influence of sporulation environment on the heat sensitivity parameter (z) (aoctool and multcompare functions, Statistics Toolbox; MATLAB 7.9.0; The Math-works). For each statistical analysis, a significant difference between compared data was conclude at the risk 5% ($\alpha=5\%$).

3 Results

3.1 Influence of the sporulation conditions on the shape of survival curves

Typical survival curves were presented in the figure 1. An average of ten survivor enumerations was performed per survival curve. The shape parameter p , estimated from the Weibull model, was dependent on the bacterial strain and on the type of sporulation media SMB or MNA (likelihood ratio test, $\alpha=0.05$). However neither the sporulation temperature, pH nor the heating temperature were found to affect the shape parameter p (likelihood ratio test, $\alpha=0.05$). Therefore survival curves were fitted by the Weibull

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model using a unique shape parameter (p) for a couple strain-sporulation medium (Table 1).

3.2 Modeling the influence of sporulation temperature on the spore heat resistance

As shown in Figure 2A, an increase of sporulation temperature led to an increase of the heat resistance (δ) of *B. weihenstephanensis* KBAB4 spores up to a maximal resistance (δ_{max}). The spore heat resistance (δ) decreased more than a tenfold when spores were produced at 5°C rather than at 30°C (Figure 2A). The sporulation temperature giving the highest heat resistance seemed close to 30°C and a higher sporulation temperature led to a decrease of the spore heat resistance. Such variations of spore heat resistance were both significant for sporulation in SMB and in MNA (Kruskal-Wallis test, $\alpha=0.05$). The sporulation of *B. weihenstephanensis* KBAB4 in SMB at 10°C, 30°C and 35°C induced a spore heat resistance ($\delta_{90^\circ C}$) estimated at 1.98 min (± 0.29), 6.00 min (± 1.11) and 2.88 min (± 2.21) respectively. Similarly, sporulation in MNA at 10°C, 30°C and 35°C led to a spore heat resistance ($\delta_{90^\circ C}$) of 0.20 min (± 0.03), 1.63 min (± 0.43) and 1.08 min (± 1.35) respectively (Table 1).

The same influence of the sporulation temperature on spore heat resistance was observed for *B. licheniformis* AD978 strain, with a sporulation temperature close to 45°C maximizing the spore heat resistance (Figure 2B). The sporulation of *B. licheniformis* AD978 in SMB at 20°C, 45°C and 50°C induced a spore heat resistance ($\delta_{100^\circ C}$) of 0.59 min (± 0.07), 3.78 min (± 0.94) and 1.61 min (± 1.03) respectively. Spores produced in MNA at 20°C were significantly less resistant than spores produced at 45°C and 50°C

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(Kruskal-Wallis test, $\alpha=0.05$) with respectively $\delta_{100^\circ\text{C}}$ equal to 1.51 min (± 0.23), 7.95 min (± 1.68) and 6.63 min (± 0.88) (Table 1).

Therefore, from the equation (5), the four parameters were estimated at 7.94 min ($\delta_{\max}$), 24.4°C ($T_{\text{opt}(R)}$), 2.4°C ($T_{\min(R)}$), and 37.4°C ($T_{\max(R)}$) for *B. weihenstephanensis* KBAB4 spores produced in SMB and at 6.02 min, 49.9°C, 5.7°C and 50.0°C respectively for *B. licheniformis* AD978 spores produced in SMB (Table 2).

3.3 Modeling the influence of sporulation pH on the spore heat resistance

As shown in Figure 2C, the more acidic the sporulation pH in SMB was, the lower the heat resistance of *Bacillus weihenstephanensis* KBAB4 spores was. Heat resistance values ($\delta_{90^\circ\text{C}}$) of spores produced in SMB at pH 5.9 (2.67 min ± 0.35) were significantly lower than those of spores produced at pH 7.2 (6.00 min ± 1.11) (Figure 2C). The characterization of heat resistance ($\delta_{100^\circ\text{C}}$) of *B. licheniformis* AD978 spores produced in SMB at pH 6.3 (2.78 min ± 0.88) and 7.2 (3.78 min ± 0.94) allowed the observation of a slight but not significant difference of heat resistance (Figure 2D).

From the resistance secondary model (Equation 5), the parameters were estimated at 7.94 min ($\delta_{\max}$), pH 8.0 ($\text{pH}_{\text{opt}(R)}$), and 5.2 ($\text{pH}_{\min(R)}$) for *B. weihenstephanensis* KBAB4 and respectively at 6.02 min, pH 8.5 and 5.6 for *B. licheniformis* AD978 (Table 2).

3.4 Independence of the model parameters towards heating temperature

No significant influence of the heating temperature on the estimation of the five environmental parameters $T_{\min(R)}$, $T_{\text{opt}(R)}$, $T_{\max(R)}$, $\text{pH}_{\min(R)}$ and $\text{pH}_{\text{opt}(R)}$ was noticed (likelihood ratio test, $\alpha=0.05$) (Figure 4A and 4B). The heating temperature did not interact with the influence of the sporulation environment on the spore heat resistance.

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Therefore the five environmental parameters were estimated from the resistance secondary model regardless of the heating temperature.

Moreover, from the individual fit of the Bigelow model (Equation 4), the temperature and pH during sporulation did not affect significantly the heat sensibility (z) of spores (Figure 3). The slope of the Bigelow log-linear relationship between the spore heat resistance and the heating temperature was constant whatever the sporulation temperature and pH. Therefore, from the resistance secondary model (Equation 5), z value was estimated at 9.0°C (± 0.4) for *B. weihenstephanensis* KBAB4 spores whatever the sporulation temperature and pH. For *B. licheniformis* AD978 spores, z value was estimated at 7.3°C (± 0.3) (Table 2). In this way, the resistance secondary model could be extended to take into account the heating temperature without the necessity of adding an interaction term between the sporulation environment and the spore heat resistance.

4 Discussion

For sub-optimal sporulation temperatures, the increase of spore heat resistance with the increase of sporulation temperature was already mentioned in numerous studies on *B. anthracis*, *B. cereus*, *B. licheniformis*, *B. subtilis* and *B. weihenstephanensis* spores (Baweja *et al.*, 2008; Condon *et al.*, 1992; Garcia *et al.*, 2010; Gonzalez *et al.*, 1999; Melly *et al.*, 2002; Planchon *et al.*, 2011; Raso *et al.*, 1995). The decrease of heat resistance of spores produced at high sporulation temperatures was already noticed for one strain of *B. subtilis* and of *B. cereus* (Gonzalez *et al.*, 1999; Lindsay *et al.*, 1990). Moreover, oppositely to the spore heat resistance (δ) and in agreement with others authors, the heat sensitivity (z) was reported to be independent of the sporulation

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temperature (Condon *et al.*, 1992; Gonzalez *et al.*, 1999; Raso *et al.*, 1995) and of the sporulation pH (Mazas *et al.*, 1997). The current observations suggest the existence, for each strain, of an optimum sporulation temperature maximizing heat resistance of spores. The log linear model proposed by Leguerinel *et al.* (2007) could therefore be valid only for a suboptimal range of sporulation temperatures. The resistance secondary model proposed in this work presents the advantage of estimating the sporulation temperature maximizing the spore heat resistance, which is of prime interest for the calculation of the process efficiency of heating processes. Moreover, in order to test its robustness, the model was challenged to data on the influence of sporulation temperature on the resistance of spores of *B. cereus* ATCC7004 published by Gonzalez *et al.* (1999) (Figure 4C). *B. cereus* ATCC7004 spores produced at 35°C were more resistant than spores produced at 20°C and 40°C (Gonzalez *et al.*, 1999). The temperature parameters ($T_{min(R)}$, $T_{opt(R)}$ and $T_{max(R)}$) were estimated from the resistance secondary model at 11.8°C, 37.7°C and 47.0°C respectively. The goodness of the model fit was illustrated by comparing the observed heat resistance values ($\log \delta$) with those calculated from the resistance secondary model (Figure 5). As shown by its robustness, the model could notably be fitted from different data sets and from spores produced in different sporulation media. It can also be emphasized that characteristic temperature values estimated from the resistance secondary model are close to growth cardinal values and reflect as well the psychrotrophic behavior for *B. weihenstephanensis* KBAB4 strain as compared to the mesophilic behavior for the *B. licheniformis* AD978 strain.

To conclude, the proposed model quantifies the variation of spore heat resistance as a function of the temperature and pH for a given sporulation environment. Moreover, it

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appears that the influence of sporulation temperature and pH acts on the spore heat resistance in the same direction as the heating and recovery temperatures and pHs (Mafart *et al.*, 2010). For instance, the decrease of spore heat resistance is favoured by acidic sporulation conditions as well as acidic treatment or acidic recovery conditions (Couvert *et al.*, 1999; Mafart *et al.*, 2001). The estimation of the sporulation temperature and pH maximizing the spore heat resistance is of great interest to produce spores to assess spore inactivation for heating processes applied by the food industry. Further researches might be useful to extend this study to anaerobic spore forming bacteria.

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Figures

Figure 1. Typical survival curves of *Bacillus* spores produced in SMB at various sporulation temperatures and pHs. (A) Spores of *B. weihenstephanensis* KBAB4 produced at 30°C pH 7.2 (black filled symbols), at 10°C pH 7.2 (grey filled symbols) or at 30°C pH 5.9 (white filled symbols) and heat-treated at 90°C. (B) Spores of *B. licheniformis* AD978 produced at 45°C pH 7.2 (black filled symbols), at 20°C pH 7.2 (grey filled symbols) or at 45°C pH 6.3 (white filled symbols) and heat treated at 100°C. Squares, triangles or circles correspond to three independent spore productions.

Figure 2. Influence of the sporulation temperature and pH on the heat resistance of *Bacillus* spores produced in SMB. On the left, heat resistance of *B. weihenstephanensis* KBAB4 spores heat treated at 90°C (A, C). On the right, heat resistance of *B. licheniformis* AD978 spores heat treated at 100°C (B, D). Symbols correspond to independent experimental data. Predicted values by equation 5 are represented as the black lines.

Figure 3. Influence of heating temperature on the heat resistance ($\log \delta$) of *Bacillus* spp. spores produced in SMB at various temperatures and pHs. (A) *B. weihenstephanensis* KBAB4 spores produced at 30°C pH 7.2 (dark filled symbols), at 10°C pH 7.2 (grey filled symbols) and at 30°C pH 5.9 (white filled symbols). (B) *B. licheniformis* AD978 spores produced at 45°C pH 7.2 (black filled symbols), at 20°C pH 7.2 (grey filled symbols) and at 45°C pH 6.3 (white filled symbols). Symbols correspond to independent

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experimental data. Predicted values obtained by equation 5 for spore heat resistance are indicated as the black lines.

Figure 4. Modeling the influence of sporulation temperature on *Bacillus* spore heat resistance for different heating temperatures. (A) *B. weihenstephanensis* KBAB4 spores produced in SMB, treated at 95°C (circles), 90°C (diamonds) and 85°C (triangles). (B) *B. licheniformis* AD978 spores produced in SMB, treated at 100°C (circles), 95°C (diamonds) and 90°C (triangles). (C) *B. cereus* ATCC7004 spores produced in modified NA (Gonzalez et al., 1999), treated at 102°C (circles), 100°C (squares), 96°C (diamonds) and 92°C (triangles). Symbols correspond to independent experimental data. Predicted values obtained by equation 5 for spore heat resistance are indicated as the black lines.

Figure 5: Comparison of the observed heat resistance parameters ($\log \delta_{obs}$) with those predicted ($\log \delta_{pred}$) from the model for *B. weihenstephanensis* KBAB4 spores produced in SMB (A), for *B. licheniformis* AD978 spores produced in SMB (B) and for *B. cereus* ATCC7004 spores produced in modified NA (C).

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sporulation process depends on sporulation temperature but not on previous cell history.

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1 Research highlights:

- 2 - A model linking sporulation temperature and pH to heat resistance is proposed
- 3 - The tested bacteria are *Bacillus weihenstephanensis* and *B. licheniformis*
- 4 - There are sporulation temperatures and pHs maximizing spore heat resistance

5

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Table 1: Examples of estimated parameters from the resistance primary model (Equation 1)

	Sporulation media	Sporulation temperature (°C)	Number of replicates	Heating temperature	Time to first decimal reduction	Shape parameter (<i>p</i>)
<i>B. weihenstephanensis</i> KBAB4	SMB	10°C	6	90°C	1.98 (± 0.29) ^a	1.25 (± 0.02) ^b
	SMB	30°C	30	90°C	6.00 (± 1.11)	1.25 (± 0.02)
	SMB	35°C	5	90°C	2.88 (± 2.21)	1.25 (± 0.02)
	MNA	10°C	3	90°C	0.20 (± 0.03)	0.71 (± 0.03)
	MNA	30°C	3	90°C	1.63 (± 0.43)	0.71 (± 0.03)
	MNA	35°C	3	90°C	1.08 (± 1.35)	0.71 (± 0.03)
<i>B. licheniformis</i> AD978	SMB	20°C	4	100°C	0.59 (± 0.07)	0.97 (± 0.03)
	SMB	45°C	8	100°C	3.78 (± 0.94)	0.97 (± 0.03)
	SMB	50°C	4	100°C	1.61 (± 1.03)	0.97 (± 0.03)
	MNA	20°C	3	100°C	1.51 (± 0.23)	1.15 (± 0.06)
	MNA	45°C	3	100°C	7.95 (± 1.68)	1.15 (± 0.06)
	MNA	50°C	3	100°C	6.63 (± 0.88)	1.15 (± 0.06)

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^a mean ($\pm$ standard deviation) calculated from estimated values of replicates; for a unique shape parameter p

^b estimated value ($\pm$ standard deviation) from the model fit; for one bacterial strain and one sporulation medium

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Table 2. Estimated parameters from the resistance secondary model (Equation 5) and quality of fit criterions.

	<i>B. weihenstephanensis</i> KBAB4	<i>B. licheniformis</i> AD978	<i>B. cereus</i> ATCC7004 (Gonzalez <i>et al.</i> , 1999)
δ^*_{max} (min)	7.9 [6.8; 9.1] ^a	6.0 [4.9; 6.0]	0.2 [0.2; 0.3]
$T_{min(R)}$ (°C)	2.4 [-1.8; 3.7]	5.7 [2.8; 8.3]	11.8 [6.6; 14.7]
$T_{opt(R)}$ (°C)	24.4 [23.2; 26.3]	49.9 [48.0; 50.0]	37.7 [35.9; 40.2]
$T_{max(R)}$ (°C)	37.4 [36.5; 39.0]	50.0 [50.0; 50.1]	47.0 [45.7; 49.2]
$pH_{min(R)}$	5.2 [4.9; 5.5]	5.6 [5.1; 5.9]	NT
$pH_{opt(R)}$	8.0 [7.4; 8.5]	8.5 [7.8; 8.5]	NT
z (°C)	9.0 [8.3; 9.7]	7.3 [6.8; 7.7]	7.8 [6.8; 9.0]
T^*_{HT} (°C)	90	100	100
n	111	56	24
SSE	3.30	1.38	0.70
RMSE	0.17	0.16	0.17

^a confidence interval at $P = 0.95$

^b NT: Not Tested

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