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

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

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

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24

25 **Abstract**

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

40

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

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**43 1 Introduction**

44 *Bacillus spp.* are widely found in food and industrial environment (Anonymous, 2005).  
45 Because of their implication in food poisoning and food spoilage, *Bacillus spp.*  
46 inactivation is a major objective in food processing (De Jonghe *et al.*, 2010; Nauta *et al.*,  
47 2003; Scheldeman *et al.*, 2005). As *Bacillus* spores are able to resist to harsh  
48 environmental conditions, the calculation of the process efficiency is necessary to ensure  
49 food safety. The variation of spore heat resistance as a function of the sporulation  
50 environment has been extensively reported. The increase in sporulation temperature leads  
51 to an increase in spore heat resistance of several *Bacillus* species (Baweja *et al.*, 2008;  
52 Garcia *et al.*, 2010; Gonzalez *et al.*, 1999; Palop *et al.*, 1999; Planchon *et al.*, 2011). At  
53 high sporulation temperatures spore resistance reaches a plateau or decreases (Gonzalez  
54 *et al.*, 1999; Lindsay *et al.*, 1990). A Bigelow-like log linear model was proposed to fit  
55 the increase of spore heat resistance; unfortunately this was not able to describe such a  
56 plateau (Leguerinel *et al.*, 2007). The increase in pH 6.0 to 8.1 in buffered sporulation  
57 media also caused a significant increase in *B. cereus* spore heat resistance (Mazas *et al.*,  
58 1997). No significant difference of heat resistance of spores formed in non-buffered  
59 media at various initial pHs was reported (Craven, 1990; Mazas *et al.*, 1997).  
60 Sporulation environment induces obvious variations of spore heat resistance, but is not  
61 taken into account in heat process calculations (Carlin, 2011). The aim of this study was  
62 to propose a model describing the spore heat resistance as a function of the sporulation  
63 environment. To test the robustness of the model, the influence of sporulation  
64 temperature and pH was studied on the spore heat resistance of the psychrotrophic  
65 species *Bacillus weihenstephanensis* and of the mesophilic species *Bacillus licheniformis*.

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67 **2 Materials and methods**68 **2.1 Bacterial strains and cell preparation**

69 *Bacillus weihenstephanensis* KBAB4 strain isolated from a forest soil (Vilas-Boas *et al.*,  
70 2002) was kindly provided by the Institut National de la Recherche Agronomique (INRA,  
71 Avignon, France). *B. weihenstephanensis* KBAB4 strain belongs to the psychrotrophic  
72 group VI of *B. cereus sensu lato* (Guinebretiere *et al.*, 2008). *Bacillus licheniformis*  
73 AD978 strain, isolated from raw dairy ingredients, was kindly provided by ADRIA  
74 Développement (Quimper, France).

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

86 **2.2 Spore production**

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

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

92 The SMB was composed of phosphate buffer ( $K_2HPO_4/KH_2PO_4$ , 39 mM) supplemented  
93 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  
94 a 0.22  $\mu m$  filter (Steritop system; Millipore Corporation, Billerica, MA). The sporulation  
95 pH ranging from pH 5.5 to 8.5 was adjusted by modifying the composition of the  
96 phosphate buffer in SMB and was checked after sterilization. To inoculate the SMB, the  
97 100 mL cell suspension was centrifuged (6000 x g, 10 min, 12°C). The pellet was  
98 resuspended in 100 mL SMB and was then incubated at the desired temperature from 5°C  
99 to 50°C under shaking at 100 rpm. When more than 95% of free spores were observed  
100 under a phase contrast microscope, the SMB was centrifuged (6000 x g, 10 min, 12°C).  
101 The spore pellet was suspended in 5 mL distilled water and stored for one month at 4°C  
102 before experimental use.

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

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

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**2.3 Determination of spore heat resistance**

112  
113 Capillary tubes of 200  $\mu$ L (Hirschman Laborgerate, Eberstadt, Germany) were filled with  
114 100  $\mu$ L of spore suspension and sealed (Baril *et al.*, 2011). Heat treatments were carried  
115 out in thermo-stated glycerol/water bath at 80°C, 85°C, 90°C, 95°C and 100°C for *B.*  
116 *weihenstephanensis* KBAB4 and at 85°C, 90°C, 95°C, 100°C and 105°C for *B.*  
117 *licheniformis* AD978. At each target heating time, one capillary tube was cooled in a  
118 water-ice bath and washed with ethanol. Extremities were blazed and broken. Decimal  
119 dilutions of the capillary tube content mixed to 0.9 mL of a rinsing solution made of  
120 tryptone salt broth (Biokar Diagnostics, Beauvais, France) were spread on nutrient agar  
121 plates using a spiral plater (WASP1; Don Whitley, Shipley, West Yorkshire, United  
122 Kingdom). Plates of *B. weihenstephanensis* KBAB4 strain were incubated at 30°C and  
123 those of *B. licheniformis* AD978 strain at 45°C for 24 h before colony enumeration. Cell  
124 concentrations were calculated and expressed as colony forming units per mL (CFU/mL).  
125 The initial spore concentration of each survival curves was determined by heating one  
126 capillary tube at 70°C for 5 min.

**2.4 Experimental design**

127  
128 The influence of the sporulation environment, temperature and pH, was studied on spore  
129 heat resistance of two bacillus species, *B. weihenstephanensis* and *B. licheniformis*. To  
130 assess the independence between the evolution of spore heat resistance and the  
131 sporulation medium, two sporulation media, SMB and MNA, were tested. The whole  
132 data sets were acquired from univariate experiments. When the sporulation temperature  
133 was studied the sporulation pH was fixed at 7.2 and when the sporulation pH was studied  
134 the sporulation temperature was fixed at 30°C for *B. weihenstephanensis* and at 45°C for  
135 *B. licheniformis*.

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

142 The influence of sporulation pH was only studied in SMB in the range of pH 5.5 to 8.5  
143 for *B. weihenstephanensis* and of pH 6.3 to 8.5 for *B. licheniformis*. At least three  
144 replicates were performed at pH 5.9 and 7.2 for *B. weihenstephanensis* and at pH 6.3 and  
145 7.2 for *B. licheniformis*.

**2.5 Models****2.5.1 Resistance primary model**

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

$$150 \log(N) = \log(N_0) - \left(\frac{t}{\delta}\right)^p \quad (1)$$

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

**2.5.2 Resistance secondary model**

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



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

$$160 \quad \delta = \delta_{max} \cdot \gamma_2(T_{spo}) \cdot \gamma_1(pH_{spo}) \quad (2)$$

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

164 Gamma is comprised between 0, at non-optimal conditions, and 1, at optimal conditions.

165 When sporulation temperature and pH are optimal, the spore heat resistance is maximal,  
166 meaning equal to  $\delta_{max}$ . The gamma modules of sporulation temperature and pH were  
167 adapted from the cardinal growth model (Rosso *et al.*, 1995), as the following equation

168 (3):

$$169 \quad \gamma_n(X_{spo}) = \begin{cases} 0.0 & X \leq X_{min(R)} \\ \frac{(X - X_{max(R)})(X - X_{min(R)})^n}{(X_{opt(R)} - X_{min(R)})^{n-1} [(X_{opt(R)} - X_{min(R)})(X - X_{opt(R)}) - (X_{opt(R)} - X_{max(R)})(n-1)X_{opt(R)} + X_{min(R)} - nX]} & X_{min(R)} < X < X_{max(R)} \\ 0.0 & X \geq X_{max(R)} \end{cases} \quad (3)$$

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

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

$$181 \quad \delta = \delta^* \cdot 10^{-\left(\frac{T_{HT} - T_{HT}^*}{z}\right)} \quad (4)$$

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

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

$$188 \quad \delta = \delta_{\max}^* \gamma_2(T_{spo}) \gamma_1(pH_{spo}) 10^{-\left(\frac{T_{HT} - T_{HT}^*}{z}\right)} \quad (5)$$

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

### 192 2.5.3 Model fit and statistical analysis

193 In order to reduce the variance of the studied responses ( $N$  and  $\delta$ ), the model parameters  
194 were estimated by minimizing the sum of squared error (SSE) of the decimal logarithm of  
195 the population size, meaning  $\log(N)$  values for the resistance primary model (Equation 1)  
196 and the decimal logarithm of the heat resistance, meaning  $\log(\delta)$  values for the resistance  
197 secondary model (Equation 5). These estimations were computed from experimental data  
198 sets by non linear regression (lsqcurvefit function, Optimization Toolbox; MATLAB  
199 7.9.0; The Math-works), as well as the estimation of the 95% confidence intervals  
200 (nlparci, Statistical Toolbox; MATLAB 7.9.0; The Math-works). The goodness of fit was

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

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

$$222 \quad S_L = n \cdot \ln \left( \frac{SSE_u}{SSE_w} \right) \quad (6)$$

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

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

### 238 3 Results

#### 239 3.1 Influence of the sporulation conditions on the shape of survival curves

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

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246 model using a unique shape parameter ( $p$ ) for a couple strain-sporulation medium (Table  
247 1).

### 248 **3.2 Modeling the influence of sporulation temperature on the spore heat** 249 **resistance**

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

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

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

270 Therefore, from the equation (5), the four parameters were estimated at 7.94 min ( $\delta_{max}$ ),  
271 24.4°C ( $T_{opt(R)}$ ), 2.4°C ( $T_{min(R)}$ ), and 37.4°C ( $T_{max(R)}$ ) for *B. weihenstephanensis* KBAB4  
272 spores produced in SMB and at 6.02 min, 49.9°C, 5.7°C and 50.0°C respectively for *B.*  
273 *licheniformis* AD978 spores produced in SMB (Table 2).

### 274 3.3 Modeling the influence of sporulation pH on the spore heat resistance

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

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

### 285 3.4 Independence of the model parameters towards heating temperature

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

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

292 Moreover, from the individual fit of the Bigelow model (Equation 4), the temperature and  
293 pH during sporulation did not affect significantly the heat sensibility ( $z$ ) of spores (Figure  
294 3). The slope of the Bigelow log-linear relationship between the spore heat resistance and  
295 the heating temperature was constant whatever the sporulation temperature and pH.  
296 Therefore, from the resistance secondary model (Equation 5),  $z$  value was estimated at  
297 9.0°C ( $\pm 0.4$ ) for *B. weihenstephanensis* KBAB4 spores whatever the sporulation  
298 temperature and pH. For *B. licheniformis* AD978 spores,  $z$  value was estimated at 7.3°C  
299 ( $\pm 0.3$ ) (Table 2). In this way, the resistance secondary model could be extended to take  
300 into account the heating temperature without the necessity of adding an interaction term  
301 between the sporulation environment and the spore heat resistance.

#### 303 4 Discussion

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

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313 temperature (Condon *et al.*, 1992; Gonzalez *et al.*, 1999; Raso *et al.*, 1995) and of the  
314 sporulation pH (Mazas *et al.*, 1997). The current observations suggest the existence, for  
315 each strain, of an optimum sporulation temperature maximizing heat resistance of spores.  
316 The log linear model proposed by Leguerinel *et al.* (2007) could therefore be valid only  
317 for a suboptimal range of sporulation temperatures. The resistance secondary model  
318 proposed in this work presents the advantage of estimating the sporulation temperature  
319 maximizing the spore heat resistance, which is of prime interest for the calculation of the  
320 process efficiency of heating processes. Moreover, in order to test its robustness, the  
321 model was challenged to data on the influence of sporulation temperature on the  
322 resistance of spores of *B. cereus* ATCC7004 published by Gonzalez *et al.* (1999) (Figure  
323 4C). *B. cereus* ATCC7004 spores produced at 35°C were more resistant than spores  
324 produced at 20°C and 40°C (Gonzalez *et al.*, 1999). The temperature parameters ( $T_{min(R)}$ ,  
325  $T_{opt(R)}$  and  $T_{max(R)}$ ) were estimated from the resistance secondary model at 11.8°C, 37.7°C  
326 and 47.0°C respectively. The goodness of the model fit was illustrated by comparing the  
327 observed heat resistance values ( $\log \delta$ ) with those calculated from the resistance  
328 secondary model (Figure 5). As shown by its robustness, the model could notably be  
329 fitted from different data sets and from spores produced in different sporulation media. It  
330 can also be emphasized that characteristic temperature values estimated from the  
331 resistance secondary model are close to growth cardinal values and reflect as well the  
332 psychrotrophic behavior for *B. weihenstephanensis* KBAB4 strain as compared to the  
333 mesophilic behavior for the *B. licheniformis* AD978 strain.  
334 To conclude, the proposed model quantifies the variation of spore heat resistance as a  
335 function of the temperature and pH for a given sporulation environment. Moreover, it



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336 appears that the influence of sporulation temperature and pH acts on the spore heat  
337 resistance in the same direction as the heating and recovery temperatures and pHs (Mafart  
338 *et al.*, 2010). For instance, the decrease of spore heat resistance is favoured by acidic  
339 sporulation conditions as well as acidic treatment or acidic recovery conditions (Couvert  
340 *et al.*, 1999; Mafart *et al.*, 2001). The estimation of the sporulation temperature and pH  
341 maximizing the spore heat resistance is of great interest to produce spores to assess spore  
342 inactivation for heating processes applied by the food industry. Further researches might  
343 be useful to extend this study to anaerobic spore forming bacteria.  
344

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351 **Figures**

352 Figure 1. Typical survival curves of *Bacillus* spores produced in SMB at various  
353 sporulation temperatures and pHs. (A) Spores of *B. weihenstephanensis* KBAB4  
354 produced at 30°C pH 7.2 (black filled symbols), at 10°C pH 7.2 (grey filled symbols) or  
355 at 30°C pH 5.9 (white filled symbols) and heat-treated at 90°C. (B) Spores of *B.*  
356 *licheniformis* AD978 produced at 45°C pH 7.2 (black filled symbols), at 20°C pH 7.2  
357 (grey filled symbols) or at 45°C pH 6.3 (white filled symbols) and heat treated at 100°C.  
358 Squares, triangles or circles correspond to three independent spore productions.  
359

360 Figure 2. Influence of the sporulation temperature and pH on the heat resistance of  
361 *Bacillus* spores produced in SMB. On the left, heat resistance of *B. weihenstephanensis*  
362 KBAB4 spores heat treated at 90°C (A, C). On the right, heat resistance of *B.*  
363 *licheniformis* AD978 spores heat treated at 100°C (B, D). Symbols correspond to  
364 independent experimental data. Predicted values by equation 5 are represented as the  
365 black lines.  
366

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

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

375  
376 Figure 4. Modeling the influence of sporulation temperature on *Bacillus* spore heat  
377 resistance for different heating temperatures. (A) *B. weihenstephanensis* KBAB4 spores  
378 produced in SMB, treated at 95°C (circles), 90°C (diamonds) and 85°C (triangles). (B) *B.*  
379 *licheniformis* AD978 spores produced in SMB, treated at 100°C (circles), 95°C  
380 (diamonds) and 90°C (triangles). (C) *B. cereus* ATCC7004 spores produced in modified  
381 NA (Gonzalez et al., 1999), treated at 102°C (circles), 100°C (squares), 96°C (diamonds)  
382 and 92°C (triangles). Symbols correspond to independent experimental data. Predicted  
383 values obtained by equation 5 for spore heat resistance are indicated as the black lines.

384  
385 Figure 5: Comparison of the observed heat resistance parameters ( $\log \delta_{obs}$ ) with those  
386 predicted ( $\log \delta_{pred}$ ) from the model for *B. weihenstephanensis* KBAB4 spores produced  
387 in SMB (A), for *B. licheniformis* AD978 spores produced in SMB (B) and for *B. cereus*  
388 ATCC7004 spores produced in modified NA (C).

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396 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

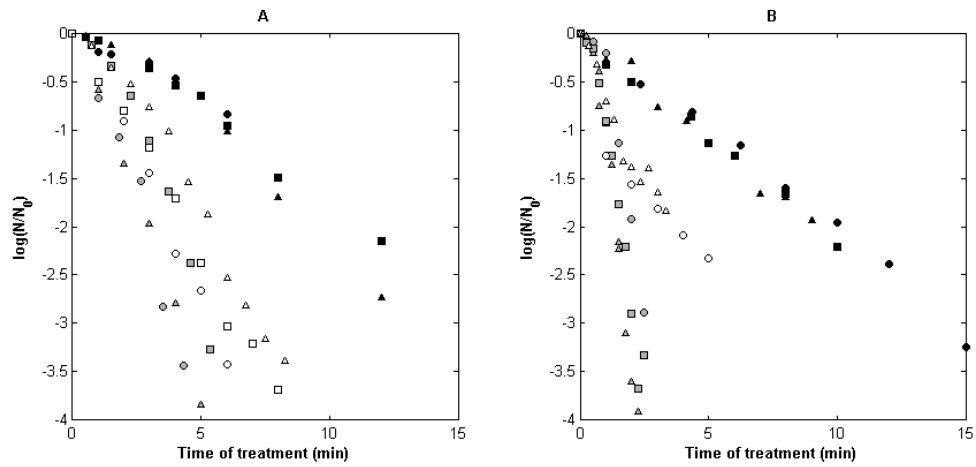
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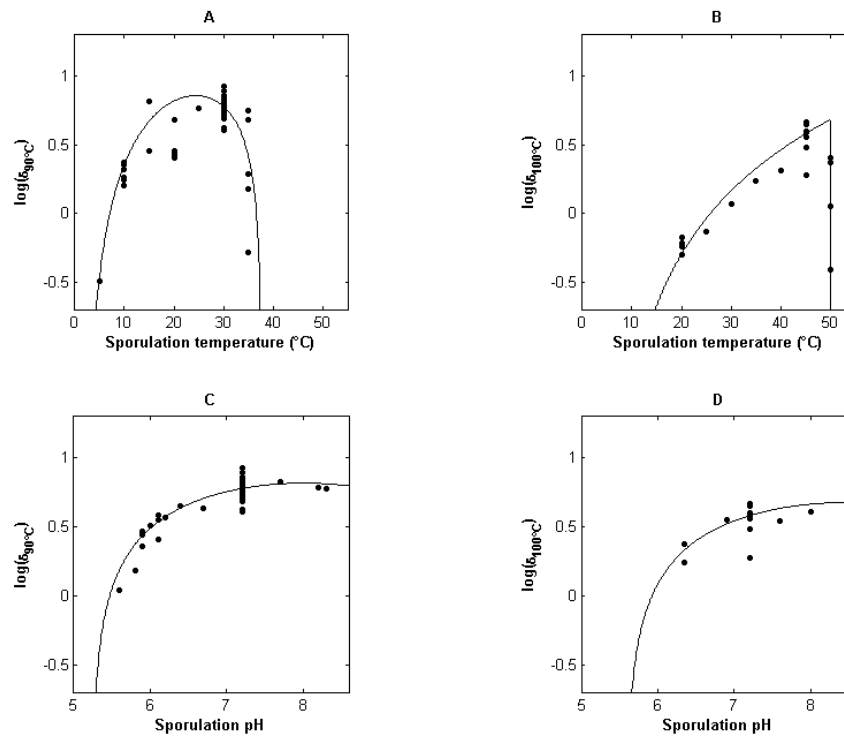
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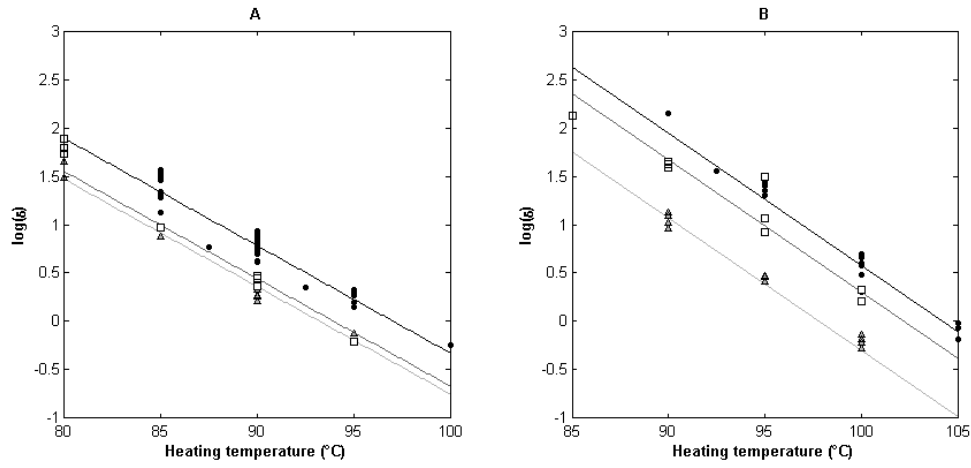
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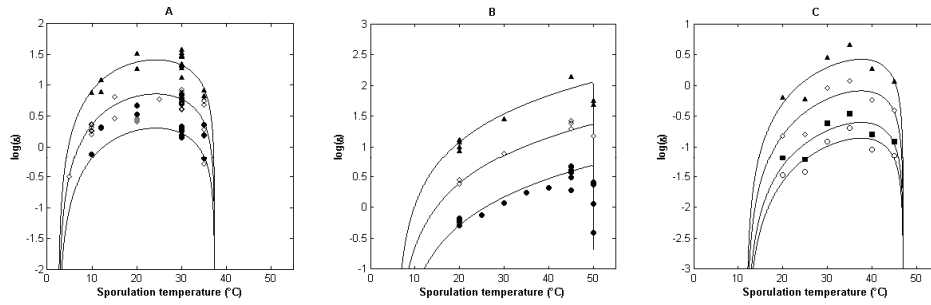
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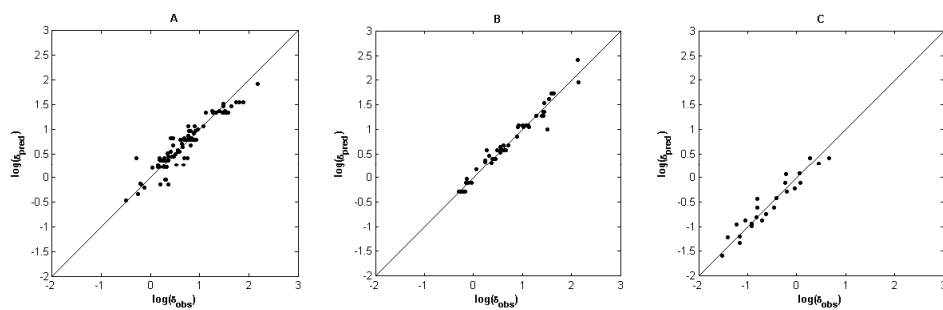
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Table 1: Examples of estimated parameters from the resistance primary model (Equation 1)

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

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

<sup>b</sup> 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)
$\delta^*_{max}$ (min)	7.9 [6.8; 9.1] <sup>a</sup>	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

<sup>a</sup> confidence interval at  $P = 0.95$

<sup>b</sup> NT: Not Tested

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