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Eugénie Baril, Louis Coroller, Olivier Couvert, Mohammed El Jabri, Ivan Leguerinel, et al.. Sporulation boundaries and spore formation kinetics of *Bacillus* spp. as a function of temperature, pH and aw.. Food Microbiology, 2012, 32 (1), pp.79-86. 10.1016/j.fm.2012.04.011 . hal-01330230

**HAL Id: hal-01330230**

**<https://hal.science/hal-01330230v1>**

Submitted on 29 May 2020

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# Accepted Manuscript



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PII: S0740-0020(12)00093-7

DOI: [10.1016/j.fm.2012.04.011](https://doi.org/10.1016/j.fm.2012.04.011)

Reference: YFMIC 1784

To appear in: *Food Microbiology*

Received Date: 8 August 2011

Revised Date: 18 April 2012

Accepted Date: 19 April 2012

Please cite this article as: Baril, E., Coroller, L., Couvert, O., El Jabri, M., Leguerinel, I., Postollec, F., Boulais, C., Carlin, F., Mafart, P., Sporulation boundaries and spore formation kinetics of *Bacillus spp.* as a function of temperature, pH and  $a_w$ , *Food Microbiology* (2012), doi: 10.1016/j.fm.2012.04.011.

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1 **Sporulation boundaries and spore formation kinetics of *Bacillus spp.* as a function of**  
2 **temperature, pH and  $a_w$**

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24 Abstract

25 Sporulation niches in the food chain are considered as a source of hazard and are not  
26 clearly identified. Determining the sporulation environmental boundaries could contribute  
27 to identify potential sporulation niches. Spore formation was determined in a Sporulation  
28 Mineral Buffer. The effect of incubation temperature, pH and water activity on time to  
29 one spore per mL, maximum sporulation rate and final spore concentration was  
30 investigated for a *Bacillus weihenstephanensis* and a *B. licheniformis* strain.

31 Sporulation boundaries of *B. weihenstephanensis* and of *B. licheniformis* were similar to,  
32 or included within, the range of temperatures, pH and water activities supporting growth.  
33 For instance, sporulation boundaries of *B. weihenstephanensis* were evaluated at 5°C,  
34 35°C, pH 5.2 and  $a_w$  0.960 while growth boundaries were observed at 5°C, 37°C, pH 4.9  
35 and  $a_w$  0.950. Optimum spore formation was determined at 30°C pH 7.2 for *B.*  
36 *weihenstephanensis* and at 45°C pH 7.2 for *B. licheniformis*. Lower temperatures and pH  
37 delayed the sporulation process. For instance, the time to one spore per mL was tenfold  
38 longer when sporulation occurred at 10°C and 20°C, for each strain respectively, than at  
39 optimum sporulation temperature. The relative effect of temperature and pH on  
40 sporulation rates and on growth rates is similar. This work suggests that the influence of  
41 environmental factors on the quantitative changes in sporulation boundaries and rates was  
42 similar to their influence on changes in growth rate.

43

44 Key words: *Bacillus weihenstephanensis*, *Bacillus licheniformis*, sporulation boundaries,  
45 growth, temperature, pH, water activity, modelling

46

47 **1 Introduction**

48 Contamination and persistence of spores of *Bacillus* and related genera in foods is a real  
49 concern for the food industry, despite a constant implementation of hygienic practices.  
50 Bacterial spores are common contaminants of food raw materials and ingredients (dairy  
51 products, flour, cocoa, bakery products, spices, meat products, vegetables or fruits, ....)  
52 (Anonymous, 2005; Carlin, 2011). In favourable conditions spores germinate, grow and  
53 may cause spoilage and quality loss. Pathogenic species may cause foodborne diseases.  
54 *B. cereus* and *B. licheniformis* for instance are well known agents of spoilage in dairy  
55 products such as sweet curdling and bitty cream (De Jonghe *et al.*, 2010; Meer *et al.*,  
56 1991). *B. cereus* emetic or diarrheal toxins are regularly at the origin of foodborne  
57 poisonings in which a large diversity of foods are implicated (Anonymous, 2005;  
58 Stenfors Arnesen *et al.*, 2008). The spore formation occurs in diverse natural or linked to  
59 agricultural and industrial activities niches (Carlin, 2011). As sources of spore  
60 contamination in the food chain, sporulation niches are potential food safety hazards. The  
61 determination of environmental conditions allowing sporulation and of sporulation  
62 boundaries may contribute to identify the main contamination sources throughout the  
63 food chain.

64 Sporulation is the latest cellular response of bacteria to nutrient depletion (Burkholder  
65 and Grossman, 2000). It generally occurs at high population density and involves cellular  
66 communication by accumulation of various extracellular oligopeptides (Auchtung and  
67 Grossman, 2008; Grossman, 1995; Sonenshein, 1999). Spore formation may be observed  
68 in laboratory with specific broth and agar media as well as in natural environments.

69 Indeed a complete life cycle of germination-growth-sporulation was observed in the  
70 mouse intestinal tract (Tam *et al.*, 2006) and thermophilic spore formation was observed  
71 in heat exchangers and evaporators (Scott *et al.*, 2007).

72 Spore formation is delayed and spore yield is generally lower at sub-optimum growth  
73 temperatures (Baweja *et al.*, 2008; Garcia *et al.*, 2010; Lechowich and Ordal, 1962;  
74 Nguyen Thi Minh *et al.*, 2011). For instance, time to achieve more than 80% of free  
75 spores of three *Bacillus cereus* strains was two to three fold longer at 20°C than at 30°C  
76 to 45°C (Gonzalez *et al.*, 1999). Similarly, acidic pH delays sporulation and reduces  
77 sporulation yield (Baweja *et al.*, 2008; Craven, 1990; Nguyen Thi Minh *et al.*, 2011;  
78 Yazdany and Lashkari, 1975). For three *Bacillus cereus* strains, time to reach 5 to 10% of  
79 free spores was at pH 6.0 two to four fold longer than time needed to achieve more than  
80 80% of free spores at pH 6.5 and 7.0 (Mazas *et al.*, 1997). These variations in sporulation  
81 time and in spore concentration were also dependent on water activity ( $a_w$ ) (Jakobsen and  
82 Murrell, 1977). At  $a_w$  0.993, 4 days were needed to obtain  $9.10^9$  spore/mL of *B. subtilis*,  
83 while 17 days were needed to form only  $3.10^7$  spore/mL at  $a_w$  0.950 (Nguyen Thi Minh *et*  
84 *al.*, 2008).

85 The aim of this work was to evaluate the influence of temperature, pH and  $a_w$  on  
86 sporulation boundaries and sporulation kinetics as follows: (i) identify the environmental  
87 conditions where sporulation could be observed, the changes in maximum spore  
88 concentration of *B. weihenstephanensis* and *B. licheniformis* were followed in two  
89 sporulation media to determine sporulation boundaries; ii) quantify the influence of  
90 environmental conditions on sporulation kinetic parameters by fitting a mathematical

91 model; and iii) compare the impact of temperature, pH and aw on sporulation boundaries  
92 and sporulation rates to those on growth boundaries and growth rates.

93

## 94 **2 Materials and Methods**

### 95 **2.1 Bacterial strains and culture conditions**

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

102 Both strains were frozen (-20°C) as 1 mL aliquots in nutrient broth diluted in 50%  
103 glycerol (v/v). A preculture was performed by diluting 1 mL aliquot into 100 mL nutrient  
104 broth (NB) (Biokar Diagnostics, Beauvais, France) in 250 mL flasks for 8 h, followed by  
105 a 1/100 preculture incubated for 16 h. Incubation was performed under shaking (100 rpm)  
106 at 30°C (45°C) for *B. weihenstephanensis* KBAB4 (for *B. licheniformis* AD978). Finally  
107 a 0.1 mL culture of *B. weihenstephanensis* KBAB4 or a 0.01 mL culture of *B.*  
108 *licheniformis* AD978 was diluted in 100 mL NB and incubated for 6 h to reach  
109 concentrations of approximately  $7.7 \log_{10}(\text{CFU/mL})$  for both strains with less than 2.6  
110  $\log_{10}(\text{CFU/mL})$  of spores, which was the limit of spore quantification (20 colonies per  
111 plates for a 50  $\mu\text{L}$  inoculation of undiluted sample).

112

113 **2.2 Determination of cardinal growth values of vegetative cells of *Bacillus sp.* by**  
114 **turbidimetry measurements**

115 Growth boundaries were considered as the minimum and maximum temperatures,  
116 minimum pH and minimum  $a_w$  values at which growth was observed (Ross *et al.*, 2011).  
117 They were observed during the estimation of cardinal growth values by using the  
118 turbidimetry method (Membré *et al.*, 2002) to compare the influence of temperature, pH  
119 and  $a_w$  on maximum growth rates. At least ten levels of temperature, pH or  $a_w$  were  
120 studied from 5°C to 55°C, from pH 4.6 to 7.5 and from  $a_w$  0.920 to 0.996, for both  
121 strains. Throughout the study, experimental data sets were collected from univariate  
122 experiments. To test the effect of one environmental factor, other factors were fixed at  
123 constant values: temperature was fixed at 30°C for *B. weihenstephanensis* KBAB4 and at  
124 45°C for *B. licheniformis* AD978, pH was fixed at 7.2 and  $a_w$  was fixed at 0.996.

125 Growth kinetics were performed in brain heart infusion supplemented with yeast extract  
126 (0.3%) and D-glucose (0.2%) (BHIYG) (all from Biokar diagnostics, Beauvais, France).  
127 The pH value was adjusted with HCl (1 or 5 N) and the  $a_w$  was adjusted with glycerol  
128 (Achard *et al.*, 1992). Broth sterilization was performed by 0.22  $\mu\text{m}$  filtration (Steritop  
129 system; Millipore Corporation, Billerica, MA). The wells of the 10x10 honeycomb plate  
130 (Oy growth curves AB Ltd; Helsinki, Finland) were filled with 200  $\mu\text{L}$  of pH-  $a_w$ -  
131 adjusted BHIYG, with one well column (10 wells) per pH or  $a_w$  condition. The vegetative  
132 cell suspension (see section 2.1) was diluted in 10 mL BHIYG to obtain a suspension of  
133 approximately 5.0  $\log_{10}$ (CFU/mL). 200  $\mu\text{L}$  of this suspension was inoculated in the first  
134 wells of each well column of the honeycomb plate. For each well column, twofold  
135 dilution series were made up from the first to the eighth well (Biesta-Peters *et al.*, 2010;



136 Membré *et al.*, 2002). The ninth and the tenth wells were used as sterility control. The  
 137 honeycomb plate was then incubated in Bioscreen C (Labsystems, Helsinki, Finland) at  
 138 the tested temperature with continuous moderate shaking. The absorbance was  
 139 automatically measured at 660 nm every 10 min. Growth at 5°C was observed by  
 140 turbidimetry measurement in 10 mL BHIYG, inoculated at approximately 5.0  
 141  $\log_{10}(\text{CFU/mL})$ . When no increase of absorbance was observed after 30 days of  
 142 incubation, no-growth was concluded.

143 The statistical analysis of Bioscreen curves was done according to previous studies  
 144 (Cuppers and Smelt, 1993; Membré *et al.*, 2002). For each well, the detection time was  
 145 determined as the time to reach the absorbance value of 0.4, meaning the middle of the  
 146 linear phase of the turbidimetry curve (Membré *et al.*, 2005). The slope of the linear  
 147 relationship between detection times and logarithm of initial concentrations corresponded  
 148 to the maximum growth rates ( $\mu_{max}$ ) (Biesta-Peters *et al.*, 2010; Le Marc *et al.*, 2002;  
 149 Membré *et al.*, 2002) (regress function, Statistical Toolbox, MATLAB 7.9.0, The Math-  
 150 works, Natick, USA). To estimate cardinal growth values, the maximum growth rates  
 151 were then fitted to the growth cardinal model according to the equation 1 (Augustin and  
 152 Carlier, 2000; Rosso *et al.*, 1995) (fminsearch function, Optimization Toolbox,  
 153 MATLAB 7.9.0, The Math-works, Natick, USA).

$$154 \quad \mu_{max} = \mu_{opt} \cdot CM_2(T) \cdot CM_1(pH) \cdot CM_1(a_w) \quad (1)$$

155 with

$$156 \quad CM_n(X) = \begin{cases} 0 & X \leq X_{min} \\ \frac{(X - X_{max})(X - X_{min})^n}{(X_{opt} - X_{min})^{(n-1)} [(X_{opt} - X_{min})(X - X_{opt}) - (X_{opt} - X_{max})((n-1)X_{opt} + X_{min} - nX)]} & X_{min} < X < X_{max} \\ 0 & X \geq X_{max} \end{cases} \quad (2)$$

158 Where  $\mu_{max}$  ( $\text{h}^{-1}$ ) is the maximum growth rate,  $\mu_{opt}$  ( $\text{h}^{-1}$ ) is the maximum growth rate at  
159 optimum conditions (optimum temperature, pH and  $a_w$ ),  $T$  ( $^{\circ}\text{C}$ ) is the temperature,  $X_{min}$ ,  
160  $X_{opt}$  and  $X_{max}$  are respectively minimum, optimum and maximum growth values.

161 As described by Efron and Tibshirani (1993) and Gauchi *et al.* (2010), the 95%  
162 confidence intervals were computed by the bootstrap resampling method of 1000 samples  
163 (bootstrap function, Statistical Toolbox, MATLAB 7.9.0, The Math-works, Natick, USA).

164

### 165 **2.3 Spore production**

166 To evaluate the dependence of the boundaries on the composition of the medium, spores  
167 were produced in Sporulation Mineral Buffer (SMB) and in Modified Nutrient Agar  
168 (MNA). Both media were prepared at various initial pH and  $a_w$  levels and incubated at  
169 various temperatures. In particular SMB is a buffered medium, which allows the control  
170 of pH during the sporulation process (Baril *et al.*, 2012). SMB was composed of  
171 phosphate buffer ( $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ , 39 mM) (Sigma-Aldrich, Saint Quentin Fallavier,  
172 France) supplemented with  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (8.0 mg/L) and  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  (1.5 mg/L)  
173 (Sigma-Aldrich, Saint Quentin Fallavier, France) (Baril *et al.*, 2011). pH was adjusted by  
174 modifying the acid-base balance of the buffer and  $a_w$  was adjusted with glycerol (Achard  
175 *et al.*, 1992). SMB was then sterilized by filtration on a 0.22  $\mu\text{m}$  filter. The vegetative cell  
176 cultures of *B. weihenstephanensis* KBAB4 and *B. licheniformis* AD978 (see section 2.1)  
177 were centrifuged at 6000 x g for 10 min at 12 $^{\circ}\text{C}$ . The pellets were suspended in 100 mL  
178 SMB. The final concentration was approximately 7.7  $\log_{10}\text{CFU/mL}$ , with less than 2.6  
179  $\log_{10}\text{CFU/mL}$  of spores. The SMB flasks were then incubated at the tested temperatures  
180 and shaken at 100 rpm.

181 The MNA was composed of nutrient agar (Biokar diagnostics, Beauvais, France)  
182 modified by the supplementation with  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (100.0 mg/L) and  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  (50.0  
183 mg/L) (Gaillard *et al.*, 1998). The pH was adjusted with HCl (1 or 5 N) and  $a_w$  with  
184 glycerol (Achard *et al.*, 1992). The MNA was then sterilized by autoclaving at 120°C for  
185 15 min. MNA plates were inoculated with 0.5 mL of the vegetative cell cultures (see  
186 section 2.1) and were incubated at the tested temperatures.

187 Spores of both strains were produced from univariate experiments at temperatures  
188 ranging from 5°C to 40°C for *B. weihenstephanensis* KBAB4 (5°C to 50°C for *B.*  
189 *licheniformis* AD978), at pH ranging from 5.2 to 8.5 and at  $a_w$  ranging from 0.935 to  
190 0.996. The pH and  $a_w$  of sporulation media were checked immediately after the  
191 inoculation respectively by a pH-meter (PHM210, MeterLab; Radiometer Copenhagen)  
192 and an  $a_w$ -meter (FA-st1 GBX; France Scientific Instrument, Romans, France).

193

#### 194 **2.4 Determination of sporulation boundaries and kinetics of *Bacillus sp.***

195 Total counts corresponded in this work to the total viable microbial population, which is  
196 vegetative cells plus spores. Total counts were performed on unheated suspension. Spores  
197 were defined as resisting cells to a heat treatment at 70°C for 5 min (Baril *et al.*, 2011).

198 To enumerate spores, a 1 mL suspension was heated at 70°C for 5 min in a glass tube by  
199 immersion into a water bath. Diluted suspensions were then spread on nutrient agar plates  
200 using a spiral plater (WASP1; Don Whitley, Shipley, West Yorkshire, United Kingdom).

201 Plates were incubated for 24 h at 30°C for *B. weihenstephanensis* and at 45°C for *B.*  
202 *licheniformis*. As mentioned by the manufacturer, the limit of quantification corresponds

203 to 20 colonies per plates for a 50  $\mu$ L inoculation of the least diluted sample, i.e. 2.6  
204  $\log_{10}$ (CFU/mL), for both total counts and spore counts.

205 Sporulation boundaries were considered as the minimum and maximum temperatures,  
206 minimum pH and minimum  $a_w$  values at which spores were detected. To determine the  
207 sporulation boundaries, final spore concentrations were evaluated at various  
208 temperatures, pH and  $a_w$  in both sporulation media. The study was performed as  
209 univariate experiments for both microorganisms at temperatures ranging from 5°C to  
210 40°C for *B. weihenstephanensis* KBAB4 (5°C to 50°C for *B. licheniformis* AD978) with  
211 a 5°C interval, at pH ranging from 5.2 to 8.5 with a 0.3 pH unit interval and at  $a_w$  ranging  
212 from 0.935 to 0.996 with a 0.005  $a_w$  unit interval. When at least 95% of free spores were  
213 observed in the sporulation medium under phase contrast microscope (Olympus BX50,  
214 Olympus Optical Co., Ltd, Hamburg, Germany), total counts and spore counts were  
215 enumerated in 2 mL aliquots of SMB, or after scrapping MNA plates and suspending  
216 spores in 5 mL of distilled water. When only a few cells were observed under phase  
217 contrast microscope, meaning concentration lower than 6  $\log_{10}$  CFU/mL, total counts and  
218 spore counts were performed after 30 days of incubation. pH and  $a_w$  values of the  
219 sporulation media were checked after microbial counting.

220 To quantify the influence of temperature and pH on spore formation, sporulation kinetics  
221 were performed in SMB at two temperature and pH levels. Sporulation kinetics were  
222 performed, as univariate experiments, at optimum conditions of temperature and pH  
223 (30°C and pH 7.2 for *B. weihenstephanensis*, 45°C and pH 7.2 for *B. licheniformis*) and  
224 at suboptimum conditions (10°C or pH 5.9 for *B. weihenstephanensis*, 20°C or pH 6.3 for  
225 *B. licheniformis*). Total counts and spore counts were performed at appropriate time

226 intervals. Three independent (different dates of experiment) sporulation kinetics were  
 227 done for each tested temperature and pH condition.

228 The following sporulation kinetic model, inspired from a logistic model using a  
 229 hyperbolic tangent function, was proposed to fit sporulation curves:

$$230 \quad \log_{10}(N_s) = \log_{10}(N_{sf}) \frac{1 - \exp(-\mu_s(t - t_{1s}))}{1 + \exp(-\mu_s(t - t_{1s}))}, t > t_{1s} \quad (3)$$

231 where  $N_s$  is the spore concentration (CFU/mL) at the time  $t$  (h),  $N_{sf}$  is the final spore  
 232 concentration (CFU/mL),  $\mu_s$  is the maximum sporulation rate ( $\text{h}^{-1}$ ) and  $t_{1s}$  is the  
 233 incubation time (h) at which  $N_s$  is theoretically equal to one spore per mL.

234 Model parameters were estimated by minimizing the sum of squared errors (SSE)  
 235 between the logarithm of the observed and the estimated spore concentration ( $\log_{10}(N_s)$ ).

236 These estimations were computed by non linear regressions (lsqcurvefit function,  
 237 Optimization Toolbox, MATLAB 7.9.0, The Math-works, Natick, USA), as well as the

238 estimation of the 95% confidence intervals (nlparci, Statistical Toolbox, MATLAB 7.9.0,  
 239 The Math-works, Natick, USA). Since the estimated values of the three replicates were

240 comparable for all environmental conditions, sporulation kinetics were fitted once for  
 241 each triplicate experimental data set to estimate only one set of parameters per

242 environmental condition (i.e.  $\log_{10}(N_{sf})$ ,  $\mu_s$  and  $t_{1s}$ ). The goodness of the model fit was  
 243 checked by the root mean square error (RMSE) (Equation 4).

$$244 \quad RMSE = \sqrt{\frac{SSE}{n - p}} \quad (4)$$

245 where  $SSE$  is the sum of squared errors,  $n$  is the number of experimental data set and  $p$  is  
 246 the number of estimated parameters.

247 The Kruskal-Wallis test was computed to test the influence of the sporulation  
248 temperature, pH and  $a_w$  on final spore concentrations and on estimated parameters  
249 (kruskalwallis and ranksum functions, Statistics Toolbox, MATLAB 7.9.0; The Math-  
250 works). For each statistical analysis, a significant difference between compared data was  
251 stated at the risk  $\alpha$  of 5%.

### 252 **3 Results**

#### 253 **3.1 Growth abilities**

254 Growth of *B. weihenstephanensis* KBAB4 was observed from 5°C to 37°C, however at  
255 37°C growth was only observed for one out of two experiments (Figure 1A). Growth was  
256 observed from pH 4.9 to 7.5, and not at pH 4.6 and 4.8 (Figure 1C). Growth was also  
257 observed from  $a_w$  0.950 to 0.996, and not at  $a_w$  0.920, 0.930 and 0.940 (Figure 1E). Thus  
258 growth boundaries of *B. weihenstephanensis* KBAB4 were at 5°C, 37°C, pH 4.9 and  $a_w$   
259 0.950. Growth cardinal values of *B. weihenstephanensis* KBAB4 were estimated at  
260 0.9°C, 33.1°C and 38.6°C, respectively for minimum, optimum and maximum  
261 temperatures, at pH 4.6 and  $a_w$  0.946 respectively for minimum growth pH and  $a_w$ . The  
262 maximum growth rate ( $\mu_{max}$ ) was estimated at 1.68 h<sup>-1</sup> (Table 1).

263 Growth of *B. licheniformis* AD978 was observed from 20°C to 55°C. No growth at 7°C,  
264 10°C and 15°C was observed (Figure 1B). Growth was also observed from pH 4.9 to 7.5  
265 (Figure 1D) and from  $a_w$  0.920 to 0.996 (Figure 1F). Growth boundaries of *B.*  
266 *licheniformis* AD978 were at 20°C, 55°C, pH 4.9 and  $a_w$  0.920. Growth cardinal values  
267 estimated from the equation 1 were 8.6°C, 50.9°C, 56.1°C for minimum, optimum,  
268 maximum temperatures, at pH 5.3 and  $a_w$  0.915 respectively for minimum pH and  $a_w$ .  
269 The maximum growth rate was estimated at 3.12 h<sup>-1</sup> (Table 1).

## 270 **3.2 Sporulation abilities**

### 271 **Effect of temperature on sporulation abilities**

272 For *B. weihenstephanensis*, the average final spore concentration in SMB was slightly but  
273 significantly higher at 30°C (7.3 log<sub>10</sub>CFU/mL) than at 10°C and 35°C (6.3 log<sub>10</sub>CFU/mL  
274 and 6.4 log<sub>10</sub>CFU/mL, respectively) ( $p < 0.05$ ) (Figure 2A). Out of this range, the final  
275 spore concentration largely decreased. At 5°C, spores were formed in only one out of  
276 three replicates indicating that the sporulation temperature boundary was reached.  
277 Similarly, at 40°C, no spore formation was detected. For *B. licheniformis*, the final spore  
278 concentration in SMB was not significantly different between 20°C and 45°C ( $p > 0.05$ )  
279 and was comprised between 6.9 and 7.5 log<sub>10</sub>CFU/mL (Figure 2B). Out of this range, a  
280 sharp decrease in the final spore concentration was observed. At 5°C, 10°C and 15°C, no  
281 spore was detected. At 50°C, the final spore concentration of 4.6 log<sub>10</sub>CFU/mL was  
282 significantly lower than at 45°C ( $p < 0.05$ ).

283 The same trend was observed in MNA for both strains. Sporulation temperature  
284 boundaries ranged from 5°C to 35°C for *B. weihenstephanensis* KBAB4 and from 20°C  
285 to 50°C for *B. licheniformis* AD978. Therefore, sporulation boundaries are close to  
286 temperature growth boundaries, which were observed at 5°C and 37°C for *B.*  
287 *weihenstephanensis* KBAB4 and from 20°C to 50°C for *B. licheniformis* AD978.

288

### 289 **Effect of pH on sporulation abilities**

290 Variations of the pH along the sporulation periods were less pronounced in SMB than in  
291 MNA. In SMB, the pH increased of 0.4 units for an initial pH of 5.2 and decreased of 0.2  
292 units for an initial pH of 8.5. Lower variations were observed at initial pH between 5.6

293 and 8.0. In MNA, whatever the initial sporulation pH was, ranging from 5.6 to 7.2, the  
294 final sporulation pH was measured at 8.7 ( $\pm 0.1$ ).

295 For *B. weihenstephanensis* KBAB4, the final spore concentrations in SMB were not  
296 different from pH 8.5 to 7.2 and were close to 7.0 log<sub>10</sub>CFU/mL (Figure 2C). The final  
297 spore concentration decreased progressively as pH decreased. For example, no significant  
298 differences were noticed between the final spore concentration at pH 7.2 and 5.9. and  
299 between pH 5.9 and 5.6 ( $p > 0.05$ ), however significant differences were observed  
300 between the final spore concentration at pH 7.2 and 5.6 ( $p < 0.05$ ). The final spore  
301 concentration decreased sharply from 6.4 log<sub>10</sub>CFU/mL at pH 5.6 to 4.0 log<sub>10</sub>CFU/mL at  
302 pH 5.2. For *B. licheniformis* AD978, the final spore concentration in SMB remained  
303 unchanged from pH 8.5 to 7.0 and reached 7.5 log<sub>10</sub>CFU/mL (Figure 2D). At pH 6.3 the  
304 final spore concentration decreased significantly to 5.0 log<sub>10</sub>CFU/mL ( $p < 0.05$ ). No  
305 spore was detected at pH 5.2, 5.5 and 5.8. In MNA, final spore concentrations were not  
306 significantly different from pH 5.6 to 7.2 for *B. weihenstephanensis* KBAB4 and from pH  
307 6.3 to 7.2 for *B. licheniformis* AD978 ( $p > 0.05$ ). Sporulation pH boundaries in SMB  
308 were evaluated at pH 5.2 for *B. weihenstephanensis* KBAB4 and at pH 5.8 for *B.*  
309 *licheniformis* AD978. These sporulation boundaries are higher than the growth  
310 boundaries for pH (at pH 4.9 for both strains).

311

### 312 **Effect of $a_w$ on sporulation abilities**

313 In SMB, no significant difference of  $a_w$  values was observed from the beginning to the  
314 end of sporulation period. In MNA,  $a_w$  values increased during the incubation. The  
315 maximum increase (0.034  $a_w$  unit) was observed at an initial  $a_w$  of 0.955.



316 For *B. weihenstephanensis* KBAB4, the final spore concentration in SMB increased  
317 significantly from 3.2 log<sub>10</sub>CFU/mL to 7.3 log<sub>10</sub>CFU/mL as a<sub>w</sub> increased from 0.965 and  
318 0.996 ( $p < 0.05$ ) (Figure 2E). At a<sub>w</sub> 0.960, spores were formed in one out of two  
319 replicates. For *B. licheniformis* AD978, the final spore concentration in SMB increased  
320 largely from 3.1 to 7.5 log<sub>10</sub>CFU/mL between a<sub>w</sub> 0.985 and 0.996 ( $p < 0.05$ ) (Figure 2F).  
321 At a<sub>w</sub> 0.975 and a<sub>w</sub> 0.980, spores were detected in one out of two or three replicates. The  
322 same trend was observed in MNA, but on a wider range of a<sub>w</sub> for both strains comprised  
323 between a<sub>w</sub> 0.935 and 0.996. The sporulation a<sub>w</sub> boundaries were in SMB at a<sub>w</sub> 0.960 for  
324 *B. weihenstephanensis* KBAB4 and at 0.975 for *B. licheniformis* AD978. These  
325 sporulation boundaries are higher than the growth boundaries being respectively 0.950  
326 and 0.920 for each strain.

327

### 328 **3.3 Sporulation kinetics**

329 Because the a<sub>w</sub> range allowing spore formation was too narrow (between 0.960 and 0.996  
330 for *B. weihenstephanensis* and between 0.975 and 0.996 for *B. licheniformis*) and final  
331 spore concentration was close to the limit of quantification, sporulation kinetics were not  
332 performed at different a<sub>w</sub> levels.

333 As shown on Figure 3, equation 3 gave a good description of the observed spore  
334 concentration and allowed the quantification of the time to achieve one spore per mL  
335 ( $t_{1S}$ ), the maximum sporulation rate ( $\mu_s$ ) and the final spore concentration ( $N_{sf}$ ).

336 As previously observed, the final spore concentrations of *B. weihenstephanensis* KBAB4  
337 were significantly lower at 10°C than at 30°C while no significant difference was  
338 observed between pH 5.9 and 7.2 ( $p > 0.05$ ) (Table 2). The time to observe one spore per

339 mL ( $t_{IS}$ ) of *B. weihenstephanensis* KBAB4 was estimated at 148.0 h, 14.5 h and 6.7 h  
340 respectively at 10°C pH 7.2, at 30°C pH 5.9 and at 30°C pH 7.2 (Table 2). This time ( $t_{IS}$ )  
341 was significantly prolonged when temperature and pH decreased from 30°C to 10°C and  
342 from pH 7.2 to 5.9 ( $p < 0.05$ ). The maximum sporulation rate ( $\mu_S$ ) was significantly lower  
343 at 10°C ( $0.05 \text{ h}^{-1}$ ) than at 30°C ( $0.60 \text{ h}^{-1}$ ) but was not significantly different between pH  
344 5.9 ( $0.50 \text{ h}^{-1}$ ) and pH 7.2 ( $0.60 \text{ h}^{-1}$ ) ( $p > 0.05$ ).

345 For *B. licheniformis* AD978, the final spore concentration was no significantly different  
346 between 20°C and 45°C but was significantly lower at pH 6.3 than at pH 7.2 (Table 2) ( $p$   
347  $< 0.05$ ).  $t_{IS}$  was estimated at 45.9 h, 4.2 h and 1.6 h for sporulation at 20°C pH 7.2, at  
348 45°C pH 6.3 and at 45°C pH 7.2, respectively (Table 2).  $t_{IS}$  increased when temperature  
349 or pH decreased ( $p < 0.05$ ). The maximum sporulation rate ( $\mu_S$ ) was significantly lower at  
350 20°C than at 45°C ( $0.02 \text{ h}^{-1}$  and  $0.37 \text{ h}^{-1}$  respectively), but significantly higher at pH 6.3  
351 than at pH 7.2 ( $0.55 \text{ h}^{-1}$  and  $0.37 \text{ h}^{-1}$  respectively) ( $p < 0.05$ ) (Table 2).

352

#### 353 4 Discussion

354 This study shows that the range of temperature, pH and  $a_w$  allowing sporulation of *B.*  
355 *weihenstephanensis* KBAB4 and of *B. licheniformis* AD978 is similar to, or lying within,  
356 the range of temperature, pH and  $a_w$  allowing growth. These results are in agreement with  
357 previous works. For instance, *B. sphaericus* grew from 10°C to 40°C while sporulation  
358 was only observed between 10°C and 30°C (De Pieri and Ludlow, 1992). Similarly,  
359 growth of three *B. cereus* strains was lying between pH 5.0 and pH 9.5 when their  
360 sporulation was only possible between pH 6.5 and 8.0 (Mazas *et al.*, 1997).  
361 Consequently, as a first approximation, it seems that temperature, pH and  $a_w$  growth

362 boundaries could be as well used as an estimation of sporulation boundaries even if it  
363 would be a conservative estimation. For tested conditions, the final spore concentration  
364 markedly decreased as temperature, pH and  $a_w$  were close to growth boundaries (Figure 1  
365 and 2). It must be noticed that care should be taken when inferring sporulation boundaries  
366 from growth boundaries of *Bacillus* strains because differences due to strain type,  
367 physiological state of cells and composition of the media used might occur.

368 A primary model was proposed to quantify the sporulation kinetics. Spore formation was  
369 delayed as sporulation temperature and pH decreased (Figure 3). For instance, a tenfold  
370 decrease of the time to one spore per mL ( $t_{1S}$ ) was observed at 10°C as compared to 30°C  
371 for *B. weihenstephanensis* KBAB4 and at 20°C as compared to 45°C for *B. licheniformis*  
372 AD978. Although the maximum sporulation rate decreased with the sporulation  
373 temperature, such trend was not observed with the decrease of pH. The ratios between  
374 maximum sporulation rates at sub-optimum temperature and reference temperature were  
375 calculated to assess the relative effect of environmental factors on sporulation rates (Ross  
376 and Dalgaard, 2004) ( $\mu_{S10^\circ C}/\mu_{S30^\circ C}$  for *B. weihenstephanensis* for instance) (Table 3).  
377 These ratios were compared to the ratios of maximum growth rates, estimated at the same  
378 temperatures from the growth cardinal model (Equation 1) and from cardinal growth  
379 values (Table 1) ( $\mu_{G10^\circ C}/\mu_{G30^\circ C}$ ). For *B. weihenstephanensis* KBAB4 cells, the ratio of  
380 maximum growth rates was evaluated at 0.12 for temperature, and that of sporulation  
381 rates was evaluated at 0.08. The ratios of maximum growth rates and sporulation rates  
382 were also calculated for the pH. For *B. weihenstephanensis* KBAB4, these ratios were  
383 respectively evaluated at 0.82 ( $\mu_{GpH5.9}/\mu_{GpH7.2}$ ), and at 0.83 ( $\mu_{SpH5.9}/\mu_{SpH7.2}$ ). For *B.*  
384 *licheniformis* AD978, same trends were observed with a lower growth ratio

385 ( $\mu_{GpH6.3}/\mu_{GpH7.2}=0.92$ ) than sporulation ratio ( $(\mu_{SpH6.3}/\mu_{SpH7.2}=1.49)$ ). The slope of the  
386 linear regression between the ratio on  $\mu_S$  values and the ratio on  $\mu_G$  values was not  
387 significantly different of 1 (t-test,  $p > 0.1$ ). Even though a model validation at  
388 intermediate growth and sporulation conditions is certainly necessary, these results  
389 suggest that in a general way the quantitative influence of temperature and pH on growth  
390 and sporulation are highly similar. As a consequence, as a first approximation, the  
391 knowledge of factors determining growth of spore forming bacteria may also be used for  
392 the determination of environmental conditions favorable to spore formation and for the  
393 determination of sporulation rates.

394 Food processes may induce prolonged residence times of food material and therefore a  
395 higher exposure probability of spore-forming bacteria to environments potentially  
396 favourable to cells multiplication or spore formation in-line (Oomes *et al.*, 2007). To  
397 prevent spore formation, it is recommended to limit the niches where growth of *Bacillus*  
398 *sp.* could occur. Determining sporulation kinetics, either by specific experiments or from  
399 growth parameters (cardinal growth temperatures, pH or  $a_w$  for instance) may contribute  
400 to the identification of critical points in processing lines, and finally to a better control of  
401 spore-forming bacteria in foods.

402

#### 403 **Acknowledgments**

404 This work was supported by the Agence Nationale de la Recherche (ANR) (France) as  
405 part of an ANR-07-PNRA-027-07 MEMOSPORE contract, by the industrial association  
406 BBA (Bretagne Biotechnologies Alimentaires) and by the French National Association of  
407 the Technical Research (ANRT).

408

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561

562 **Figures**

563 Figure 1. Effect of temperature, pH and  $a_w$  on the maximum growth rate of *B.*  
564 *weihenstephanensis* KBAB4 (A, C, E) and on *B. licheniformis* AD978 (B, D, F). Filled  
565 symbols correspond to growth observation. Empty symbols correspond to no growth. The  
566 lines correspond to the estimation of maximum growth rate by the equation 1, which was  
567 fitted only on growth observation.

568

569 Figure 2. Effect of sporulation temperature, pH and  $a_w$  on the final spore concentration of  
570 *B. weihenstephanensis* KBAB4 strain (A, C, E) and on *B. licheniformis* AD978 strain (B,  
571 D, F). Filled symbols correspond to sporulation in SMB, empty symbols correspond to  
572 sporulation in MNA. The dashed lines correspond to the limit of quantification ( $2.6$   
573  $\log_{10}$ CFU/mL). Symbols under these lines correspond to undetectable spore  
574 concentrations.

575

576 Figure 3. Influence of the sporulation temperature and pH on sporulation kinetics of *B.*  
577 *weihenstephanensis* (A, C,) and *B. licheniformis* (B, D) in SMB. (A) Sporulation of *B.*  
578 *weihenstephanensis* at 10°C (filled symbols) and 30°C (empty symbols). (C) Sporulation  
579 of *B. weihenstephanensis* at pH 5.9 (filled symbols) and pH 7.2 (empty symbols). (B)  
580 Sporulation of *B. licheniformis* at 20°C (filled symbols) and 45°C (empty symbols). (D)  
581 Sporulation of *B. licheniformis* at pH 6.3 (filled symbols) and pH 7.2 (empty symbols).  
582 Bars represent standard deviation of the three independent triplicates. Lines correspond to  
583 the estimated spore concentrations from the sporulation kinetic model (Equation 3).  
584 Dashed lines correspond to the limit of quantification.

- 1 Table 1. Estimated cardinal growth parameters of *B. weihenstephanensis* KBAB4 and *B.*  
 2 *licheniformis* AD978 and quality of fit criterions.

	<i>B. weihenstephanensis</i> KBAB4	<i>B. licheniformis</i> AD978
$\mu_{max}$	1.68 [1.52 ; 2.19]*	3.12 [2.77 ; 3.90]
$T_{min}$	0.9 [-2.5 ; 5.9]	8.6 [2.7 ; 19.4]
$T_{opt}$	33.1 [31.9 ; 33.9]	50.9 [49.2 ; 52.6]
$T_{max}$	38.6 [37.8 ; 43.0]	56.1 [55.3 ; 68.9]
$pH_{min}$	4.6 [4.5 ; 4.8]	5.3 [5.2 ; 5.3]
$pH_{opt}$	6.9 [6.7 ; 7.1]	6.9 [6.7 ; 7.1]
$a_{wmin}$	0.946 [0.943 ; 0.950]	0.915 [0.908 ; 0.927]
$a_{wopt}$	1.000 [0.995 ; 1.000]	1.000 [0.994 ; 1.000]
Number of data**	45	48
RMSE	0.073	0.190

- 3 \* Estimated value [confidence interval of the estimated parameter value at 95%]  
 4 \*\* Number of data fitted to the model. Data corresponding to no-growth were excluded to  
 5 the fit.

- 1 Table 2. Estimated sporulation kinetic parameters of *B. weihenstephanensis* KBAB4 and of *B. licheniformis* AD978 and quality of fit  
 2 criteria (Equation 3).

	<i>B. weihenstephanensis</i> KBAB4			<i>B. licheniformis</i> AD978		
Temperature	10°C	30°C*	30°C	20°C	45°C*	45°C
pH	7.2	7.2*	5.9	7.2	7.2*	6.3
$t_{1S}$	148.0 [140.7 ; 155.3]** <sup>a</sup>	6.7 [6.3 ; 7.0] <sup>b</sup>	14.5 [13.6 ; 15.3] <sup>c</sup>	45.9 [34.2 ; 57.5] <sup>d</sup>	1.6 [1.2 ; 2.1] <sup>e</sup>	4.2 [3.7 ; 4.7] <sup>f</sup>
$\mu_S$	0.05 [0.04 ; 0.07] <sup>a</sup>	0.60 [0.50 ; 0.70] <sup>b</sup>	0.50 [0.35 ; 0.64] <sup>b</sup>	0.02 [0.02 ; 0.03] <sup>d</sup>	0.37 [0.32 ; 0.42] <sup>e</sup>	0.55 [0.45 ; 0.66] <sup>f</sup>
$\text{Log}_{10}(N_{SF})$	6.5 [6.3 ; 6.7] <sup>a</sup>	7.5 [7.3 ; 7.6] <sup>b</sup>	7.7 [7.4 ; 8.0] <sup>b</sup>	6.8 [6.6 ; 7.1] <sup>d</sup>	7.1 [6.9 ; 7.3] <sup>d</sup>	5.5 [5.3 ; 5.6] <sup>e</sup>
Number of data	22	51	26	54	89	84
RMSE	0.380	0.371	0.373	0.226	0.549	0.328

3 \* Reference sporulation temperature and pH

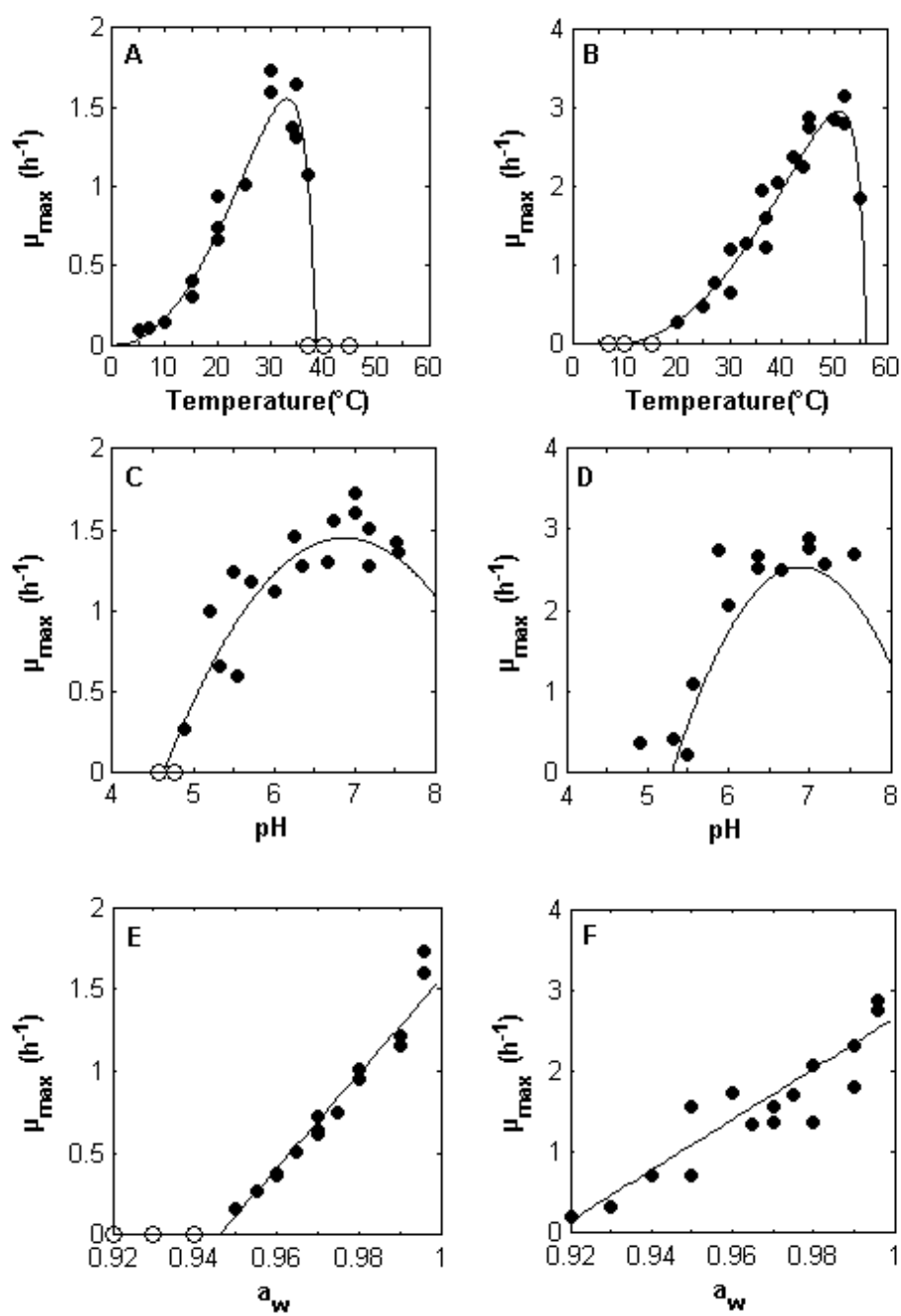
4 \*\* Estimated value [confidence interval of the estimated parameter value at 95%]

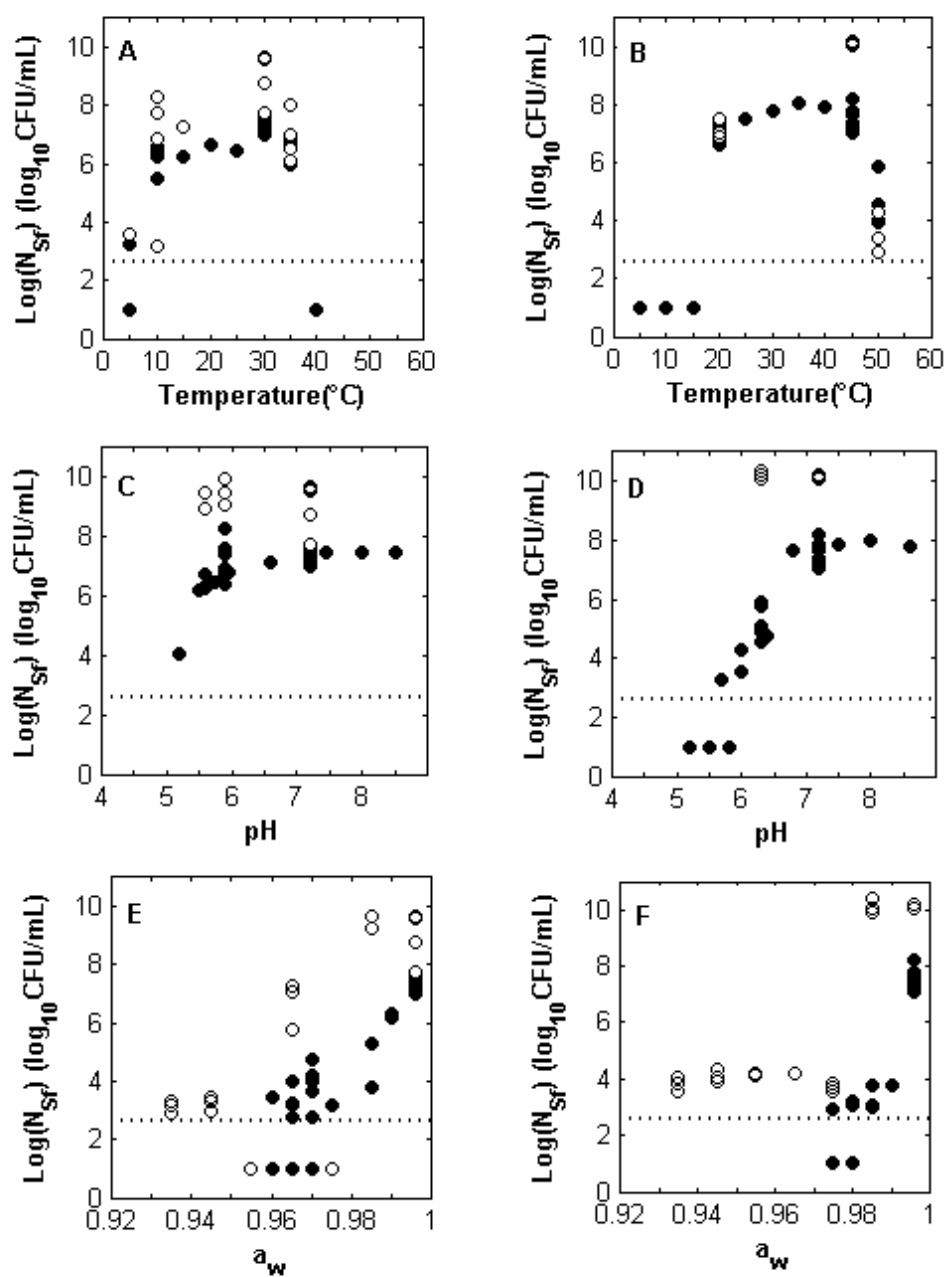
5 <sup>a-f</sup> For each line and each strain, the same superscript letter indicates that the parameter values are not significantly different ( $p > 0.05$ )

- 1 Table 3. Ratio of maximum growth rates and sporulation rates of *B. weihenstephanensis*
- 2 KBAB4 and *B. licheniformis* AD978 at different temperatures and pH.

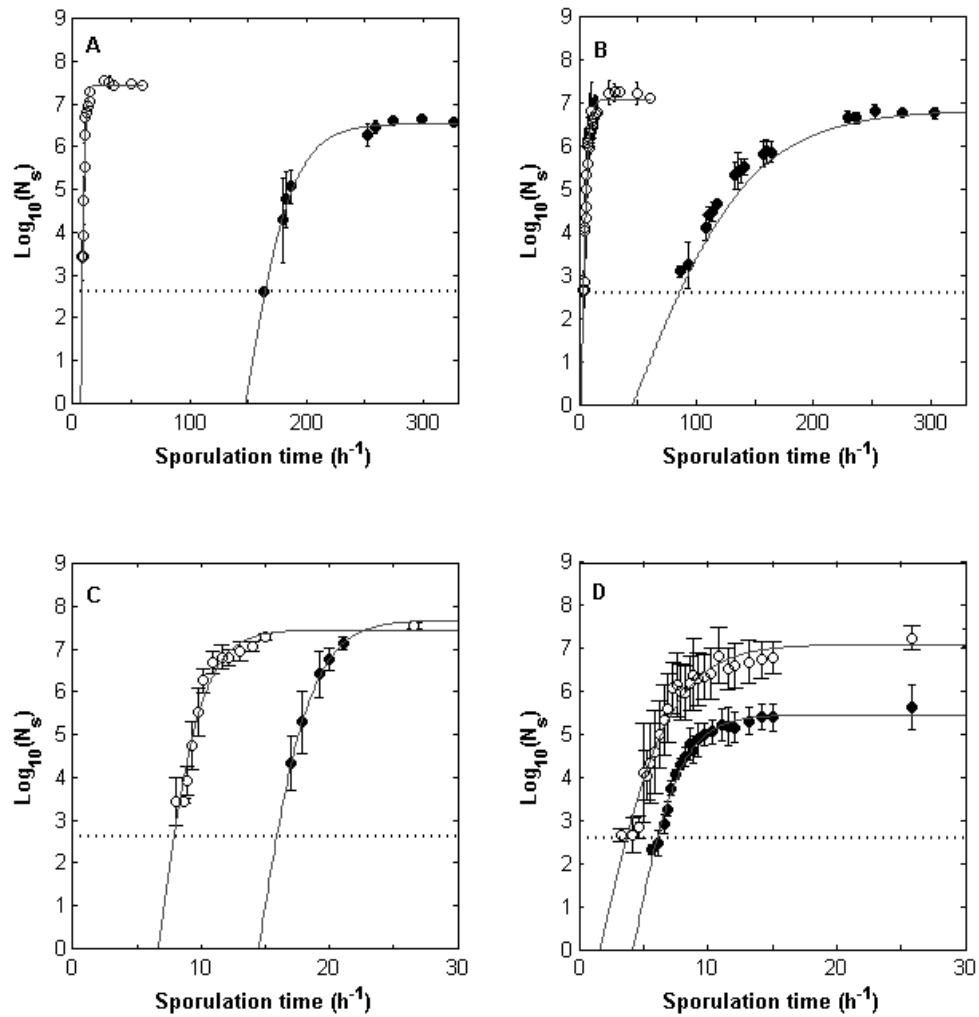
	<i>B. weihenstephanensis</i> KBAB4		<i>B. licheniformis</i> AD978	
	$\mu_{10^{\circ}\text{C}} / \mu_{30^{\circ}\text{C}}$	$\mu_{\text{pH } 5.9} / \mu_{\text{pH } 7.2}$	$\mu_{20^{\circ}\text{C}} / \mu_{45^{\circ}\text{C}}$	$\mu_{\text{pH } 6.3} / \mu_{\text{pH } 7.2}$
Growth	0.12 [0.05; 0.17]*	0.82 [0.78; 0.87]	0.11 [0.00; 0.17]	0.92 [0.82; 1.02]
Sporulation	0.08	0.83	0.05	1.49

- 3 \* Estimated ratio [confidence interval of the estimated ratio value at 95%]





A



ACCEPTED

1 **Research highlights**

2 - Temperature, pH and  $a_w$  growth limits are reasonable predictions of sporulation

3 boundaries

4 - Temperature and pH determined time to first spore

5 - Temperature and pH determined final spore concentration

6 - Sporulation rates and growth rates are similarly affected by environmental factors

7

ACCEPTED MANUSCRIPT