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

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Abstract

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

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

Key words: *Bacillus weihenstephanensis*, *Bacillus licheniformis*, sporulation boundaries, 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.

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

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

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

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

2 Materials and Methods

2.1 Bacterial strains and culture conditions

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

Both strains were frozen (-20°C) as 1 mL aliquots in nutrient broth diluted in 50% glycerol (v/v). A preculture was performed by diluting 1 mL aliquot into 100 mL nutrient broth (NB) (Biokar Diagnostics, Beauvais, France) in 250 mL flasks for 8 h, followed by a 1/100 preculture incubated for 16 h. Incubation was performed under shaking (100 rpm) at 30°C (45°C) for *B. weihenstephanensis* KBAB4 (for *B. licheniformis* AD978). Finally a 0.1 mL culture of *B. weihenstephanensis* KBAB4 or a 0.01 mL culture of *B. licheniformis* AD978 was diluted in 100 mL NB and incubated for 6 h to reach concentrations of approximately 7.7 log₁₀(CFU/mL) for both strains with less than 2.6 log₁₀(CFU/mL) of spores, which was the limit of spore quantification (20 colonies per plates for a 50 µL inoculation of undiluted sample).

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

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

Growth kinetics were performed in brain heart infusion supplemented with yeast extract (0.3%) and D-glucose (0.2%) (BHIYG) (all from Biokar diagnostics, Beauvais, France). The pH value was adjusted with HCl (1 or 5 N) and the a_w was adjusted with glycerol (Achard *et al.*, 1992). Broth sterilization was performed by 0.22 µm filtration (Steritop system; Millipore Corporation, Billerica, MA). The wells of the 10x10 honeycomb plate (Oy growth curves AB Ltd; Helsinki, Finland) were filled with 200 µL of pH- a_w -adjusted BHIYG, with one well column (10 wells) per pH or a_w condition. The vegetative cell suspension (see section 2.1) was diluted in 10 mL BHIYG to obtain a suspension of approximately 5.0 log₁₀(CFU/mL). 200 µL of this suspension was inoculated in the first wells of each well column of the honeycomb plate. For each well column, twofold dilution series were made up from the first to the eighth well (Biesta-Peters *et al.*, 2010;

Membré *et al.*, 2002). The ninth and the tenth wells were used as sterility control. The honeycomb plate was then incubated in Bioscreen C (LabSystems, Helsinki, Finland) at the tested temperature with continuous moderate shaking. The absorbance was automatically measured at 660 nm every 10 min. Growth at 5°C was observed by turbidimetry measurement in 10 mL BHIYG, inoculated at approximately 5.0 log₁₀(CFU/mL). When no increase of absorbance was observed after 30 days of incubation, no-growth was concluded.

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

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

with

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

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

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

2.3 Spore production

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

The MNA was composed of nutrient agar (Biokar diagnostics, Beauvais, France) 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 mg/L) (Gaillard *et al.*, 1998). The pH was adjusted with HCl (1 or 5 N) and a_w with glycerol (Achard *et al.*, 1992). The MNA was then sterilized by autoclaving at 120°C for 15 min. MNA plates were inoculated with 0.5 mL of the vegetative cell cultures (see section 2.1) and were incubated at the tested temperatures.

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

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

Total counts corresponded in this work to the total viable microbial population, which is vegetative cells plus spores. Total counts were performed on unheated suspension. Spores were defined as resisting cells to a heat treatment at 70°C for 5 min (Baril *et al.*, 2011). To enumerate spores, a 1 mL suspension was heated at 70°C for 5 min in a glass tube by immersion into a water bath. Diluted suspensions were then spread on nutrient agar plates using a spiral plater (WASP1; Don Whitley, Shipley, West Yorkshire, United Kingdom). Plates were incubated for 24 h at 30°C for *B. weihenstephanensis* and at 45°C for *B. licheniformis*. As mentioned by the manufacturer, the limit of quantification corresponds

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

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

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

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

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

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

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

Model parameters were estimated by minimizing the sum of squared errors (SSE) between the logarithm of the observed and the estimated spore concentration ($\log_{10}(N_s)$). These estimations were computed by non linear regressions (lsqcurvefit function, Optimization Toolbox, MATLAB 7.9.0, The Math-works, Natick, USA), as well as the estimation of the 95% confidence intervals (nlparci, Statistical Toolbox, MATLAB 7.9.0, The Math-works, Natick, USA). Since the estimated values of the three replicates were comparable for all environmental conditions, sporulation kinetics were fitted once for each triplicate experimental data set to estimate only one set of parameters per environmental condition (i.e. $\log_{10}(N_{sf})$, μ_s and t_{1s}). The goodness of the model fit was checked by the root mean square error (RMSE) (Equation 4).

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

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

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

3 Results

3.1 Growth abilities

Growth of *B. weihenstephanensis* KBAB4 was observed from 5°C to 37°C, however at 37°C growth was only observed for one out of two experiments (Figure 1A). Growth was observed from pH 4.9 to 7.5, and not at pH 4.6 and 4.8 (Figure 1C). Growth was also observed from a_w 0.950 to 0.996, and not at a_w 0.920, 0.930 and 0.940 (Figure 1E). Thus growth boundaries of *B. weihenstephanensis* KBAB4 were at 5°C, 37°C, pH 4.9 and a_w 0.950. Growth cardinal values of *B. weihenstephanensis* KBAB4 were estimated at 0.9°C, 33.1°C and 38.6°C, respectively for minimum, optimum and maximum temperatures, at pH 4.6 and a_w 0.946 respectively for minimum growth pH and a_w . The maximum growth rate (μ_{max}) was estimated at 1.68 h⁻¹ (Table 1).

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

3.2 Sporulation abilities

Effect of temperature on sporulation abilities

For *B. weihenstephanensis*, the average final spore concentration in SMB was slightly but significantly higher at 30°C (7.3 log₁₀CFU/mL) than at 10°C and 35°C (6.3 log₁₀CFU/mL and 6.4 log₁₀CFU/mL, respectively) ($p < 0.05$) (Figure 2A). Out of this range, the final spore concentration largely decreased. At 5°C, spores were formed in only one out of three replicates indicating that the sporulation temperature boundary was reached. Similarly, at 40°C, no spore formation was detected. For *B. licheniformis*, the final spore concentration in SMB was not significantly different between 20°C and 45°C ($p > 0.05$) and was comprised between 6.9 and 7.5 log₁₀CFU/mL (Figure 2B). Out of this range, a sharp decrease in the final spore concentration was observed. At 5°C, 10°C and 15°C, no spore was detected. At 50°C, the final spore concentration of 4.6 log₁₀CFU/mL was significantly lower than at 45°C ($p < 0.05$).

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

Effect of pH on sporulation abilities

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

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

For *B. weihenstephanensis* KBAB4, the final spore concentrations in SMB were not different from pH 8.5 to 7.2 and were close to 7.0 log₁₀CFU/mL (Figure 2C). The final spore concentration decreased progressively as pH decreased. For example, no significant differences were noticed between the final spore concentration at pH 7.2 and 5.9. and between pH 5.9 and 5.6 ($p > 0.05$), however significant differences were observed between the final spore concentration at pH 7.2 and 5.6 ($p < 0.05$). The final spore concentration decreased sharply from 6.4 log₁₀CFU/mL at pH 5.6 to 4.0 log₁₀CFU/mL at pH 5.2. For *B. licheniformis* AD978, the final spore concentration in SMB remained unchanged from pH 8.5 to 7.0 and reached 7.5 log₁₀CFU/mL (Figure 2D). At pH 6.3 the final spore concentration decreased significantly to 5.0 log₁₀CFU/mL ($p < 0.05$). No spore was detected at pH 5.2, 5.5 and 5.8. In MNA, final spore concentrations were not significantly different from pH 5.6 to 7.2 for *B. weihenstephanensis* KBAB4 and from pH 6.3 to 7.2 for *B. licheniformis* AD978 ($p > 0.05$). Sporulation pH boundaries in SMB were evaluated at pH 5.2 for *B. weihenstephanensis* KBAB4 and at pH 5.8 for *B. licheniformis* AD978. These sporulation boundaries are higher than the growth boundaries for pH (at pH 4.9 for both strains).

Effect of a_w on sporulation abilities

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

For *B. weihenstephanensis* KBAB4, the final spore concentration in SMB increased significantly from 3.2 log₁₀CFU/mL to 7.3 log₁₀CFU/mL as a_w increased from 0.965 and 0.996 ($p < 0.05$) (Figure 2E). At a_w 0.960, spores were formed in one out of two replicates. For *B. licheniformis* AD978, the final spore concentration in SMB increased largely from 3.1 to 7.5 log₁₀CFU/mL between a_w 0.985 and 0.996 ($p < 0.05$) (Figure 2F). At a_w 0.975 and a_w 0.980, spores were detected in one out of two or three replicates. The same trend was observed in MNA, but on a wider range of a_w for both strains comprised between a_w 0.935 and 0.996. The sporulation a_w boundaries were in SMB at a_w 0.960 for *B. weihenstephanensis* KBAB4 and at 0.975 for *B. licheniformis* AD978. These sporulation boundaries are higher than the growth boundaries being respectively 0.950 and 0.920 for each strain.

3.3 Sporulation kinetics

Because the a_w range allowing spore formation was too narrow (between 0.960 and 0.996 for *B. weihenstephanensis* and between 0.975 and 0.996 for *B. licheniformis*) and final spore concentration was close to the limit of quantification, sporulation kinetics were not performed at different a_w levels.

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

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

mL (t_{IS}) of *B. weihenstephanensis* KBAB4 was estimated at 148.0 h, 14.5 h and 6.7 h 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}) was significantly prolonged when temperature and pH decreased from 30°C to 10°C and from pH 7.2 to 5.9 ($p < 0.05$). The maximum sporulation rate (μ_S) was significantly lower at 10°C (0.05 h^{-1}) than at 30°C (0.60 h^{-1}) but was not significantly different between pH 5.9 (0.50 h^{-1}) and pH 7.2 (0.60 h^{-1}) ($p > 0.05$).

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

4 Discussion

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

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

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

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

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

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Figures

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

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

Figure 3. Influence of the sporulation temperature and pH on sporulation kinetics of *B. weihenstephanensis* (A, C,) and *B. licheniformis* (B, D) in SMB. (A) Sporulation of *B. weihenstephanensis* at 10°C (filled symbols) and 30°C (empty symbols). (C) Sporulation of *B. weihenstephanensis* at pH 5.9 (filled symbols) and pH 7.2 (empty symbols). (B) Sporulation of *B. licheniformis* at 20°C (filled symbols) and 45°C (empty symbols). (D) Sporulation of *B. licheniformis* at pH 6.3 (filled symbols) and pH 7.2 (empty symbols). Bars represent standard deviation of the three independent triplicates. Lines correspond to the estimated spore concentrations from the sporulation kinetic model (Equation 3). 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
μ_{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_{IS}	148.0 [140.7 ; 155.3]** ^a	6.7 [6.3 ; 7.0] ^b	14.5 [13.6 ; 15.3] ^c	45.9 [34.2 ; 57.5] ^d	1.6 [1.2 ; 2.1] ^e	4.2 [3.7 ; 4.7] ^f
μ_S	0.05 [0.04 ; 0.07] ^a	0.60 [0.50 ; 0.70] ^b	0.50 [0.35 ; 0.64] ^b	0.02 [0.02 ; 0.03] ^d	0.37 [0.32 ; 0.42] ^e	0.55 [0.45 ; 0.66] ^f
$\text{Log}_{10}(N_{St})$	6.5 [6.3 ; 6.7] ^a	7.5 [7.3 ; 7.6] ^b	7.7 [7.4 ; 8.0] ^b	6.8 [6.6 ; 7.1] ^d	7.1 [6.9 ; 7.3] ^d	5.5 [5.3 ; 5.6] ^e
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 ^{a-f} 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%]

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