Modeling the Recovery of Heat-Treated *Bacillus licheniformis* Ad978 and *Bacillus weihenstephanensis* KBAB4 Spores at Suboptimal Temperature and pH Using Growth Limits

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The apparent heat resistance of spores of *Bacillus weihenstephanensis* and *Bacillus licheniformis* was measured and expressed as the time to first decimal reduction (δ value) at a given recovery temperature and pH. Spores of *B. weihenstephanensis* were produced at 30°C and 12°C, and spores of *B. licheniformis* were produced at 45°C and 20°C. *B. weihenstephanensis* spores were then heat treated at 85°C, 90°C, and 95°C, and *B. licheniformis* spores were heat treated at 95°C, 100°C, and 105°C. Heat-treated spores were grown on nutrient agar at a range of temperatures (4°C to 40°C for *B. weihenstephanensis* and 15°C to 60°C for *B. licheniformis*) or a range of pHs (between pH 4.5 and pH 9.5 for both strains). The recovery temperature had a slight effect on the apparent heat resistance, except very near recovery boundaries. In contrast, a decrease in the recovery pH had a progressive impact on apparent heat resistance. A model describing the heat resistance and the ability to recover according to the sporulation temperature, temperature of treatment, and recovery temperature and pH was proposed. This model derived from secondary mathematical models for growth prediction. Previously published cardinal temperature and pH values were used as input parameters. The fitting of the model with apparent heat resistance data obtained for a wide range of spore treatment and recovery conditions was highly satisfactory.

The multiplication of spore-forming bacteria in foods can cause poisoning and/or spoilage. The heat process applied to foods (from mild in cooked and refrigerated foods to very intense in canned or ultrahigh-temperature foods) creates a positive selection of spore-forming species of bacteria because of the high resistance of their spores (1). Therefore, control of spore-forming bacteria in foods first of all requires inactivation of dormant spores by heat (or by any other appropriate inactivation treatment). The extent of inactivation depends on a number of factors, naturally including the inactivation process intensity and more importantly the spore resistance properties at the time of treatment, which may vary with the conditions and environment of sporulation (2). Respect for the organoleptic quality of food may limit the intensity of the process and therefore the extent of spore inactivation. Controlling the recovery of surviving spores in processed food strengthens the safety and stability level achieved after the inactivation process. Recovery is a complex phenomenon, comprising germination of spores, restoration of metabolic activity in suboptimal or favorable conditions and emergence of the first vegetative cell able to multiply. The incubation temperature during storage and food pH are among factors that will deeply influence the recovery of surviving spores (3).

Spore recovery is influenced by multiple physical and (bio) chemical factors, such as temperature, pH, and water activity (a_w) and by the presence of germinants (such as amino acids, ribosides, and minerals) or enzymes, such as lysozyme (4, 7, 8, 37). The previous works cited here are mainly descriptive, and modeling attempts are rare and moreover rather unsatisfactory (6). For instance, the model of Leguérinel (4) assumed a linear effect of temperature on recovery of heat-treated spores, while most works describe maximal recovery under optimal recovery conditions. In contrast, many mathematical models predict the sole impact of

heat treatment on microorganisms (15, 24, 37). This work proposes a model describing spore recovery after heat treatment as a function of incubation temperature and pH of the recovery medium and accounting for the variations due to sporulation conditions. This model integrates conditions encountered by the spores in many food industries: spores are formed under diverse environmental conditions which remain unknown most of the time, are transferred to foods, and are inactivated by heat to a certain degree during food processing. Survivors tend to multiply during food storage. The overall impact of sporulation temperature, heat treatment intensity, and temperature and pH of recovery is generally quantified by the ability of surviving spores to form a colony on an agar plate, which results from the germination and growth restoration of the heat-treated spores. The experimental work was performed on two strains with different behaviors regarding temperature: *Bacillus weihenstephanensis*, a psychrotrophic species, and *Bacillus licheniformis*, a thermotrophic species.

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MATERIALS AND METHODS

Bacterial strains. *Bacillus weihenstephanensis* strain KBAB4 (INRA, Avignon, France), isolated from forest soil (9), and *Bacillus licheniformis* strain Ad978 (ADRIA Développement, Quimper, France), isolated from dairy ingredients, were used in this work. The strains were stored at -80°C in 1-ml aliquots of brain heart infusion (BHI; Biokar Diagnostics, Beauvais, France) mixed with 50% glycerol (vol/vol) at a concentration of approximately 2×10^6 CFU ml $^{-1}$. A 100-ml volume of BHI was inoculated with 1 ml of the stock suspensions and incubated for 8 h at optimal growth temperature (30°C for *B. weihenstephanensis* KBAB4 and 45°C for *B. licheniformis* Ad978); then, a 1-ml volume was transferred into 100 ml of BHI for 16 h of incubation at the same temperatures. Finally, 0.1 ml of the *B. weihenstephanensis* suspension and 0.01 ml of the *B. licheniformis* suspension were added to 100 ml of BHI and were incubated for 6 h. For both strains, the final cell concentration in the preculture was approximately 10^8 CFU ml $^{-1}$; the number of spores estimated by the number of cells surviving a 5-min, 70°C heat treatment was lower than 100 spores ml $^{-1}$.

Sporulation. Spores were produced through a two-step sporulation process (11). Volumes of 100 ml of the previously described preculture were centrifuged ($6,000 \times g$, 10 min, 12°C) and suspended in 100 ml of sporulation mineral buffer (SMB) made of K_2HPO_4 at 4.5 g liter $^{-1}$, KH_2PO_4 at 1.8 g liter $^{-1}$, $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ at 8.0 mg liter $^{-1}$, and $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ at 1.5 mg liter $^{-1}$ and filter sterilized using 0.2- μm -pore-size filters (11). These suspensions were incubated with shaking at 30°C and 12°C for *B. weihenstephanensis* and 45°C and 20°C for *B. licheniformis* Ad978. Spores in SMB were harvested when free spores represented more than 95% of cells at a magnification of $\times 1,000$ in phase-contrast microscopy (Olympus BX50; Olympus Optical Co., Ltd., Hamburg, Germany), i.e., after 1 to 2 days at optimal growth temperature for both strains and up to 10 days at suboptimal temperature for both strains. The spore suspensions were centrifuged ($6,000 \times g$, 10 min, 12°C). Spore pellets were suspended in 5 ml of sterile distilled water. The 5-ml suspensions were divided into 1-ml aliquots and stored for 1 month at 4°C before use. Laboratory observations consistently show that spore heat resistance does not change for at least 6 months of storage (unpublished data). The final concentrations of the stock suspensions were 10^8 spores ml $^{-1}$ for *B. weihenstephanensis* and 10^9 spores ml $^{-1}$ for *B. licheniformis*.

Heat treatment. The spores were diluted in buffered peptone water (casein peptone at 10 g liter $^{-1}$, NaCl at 5 g liter $^{-1}$, K_2HPO_4 at 4.5 g liter $^{-1}$, KH_2PO_4 at 1.8 g liter $^{-1}$, pH 7.00) to a final concentration of around 10^7 spores ml $^{-1}$. Capillary tubes (200- μl volumes) were filled with 100 μl of spore suspension, sealed, and then immersed in a water-glycerol bath maintained at 85°C , 90°C , and 95°C for *B. weihenstephanensis* and 95°C , 100°C , and 105°C for *B. licheniformis* (10, 11). Capillary tubes were removed from the bath at appropriate time intervals and immediately cooled in a water-ice bath for 30 s. The tips were broken, and the heat-treated spore suspensions were diluted in tryptone salt broth (Biokar Diagnostics, Beauvais, France). To estimate the spore concentration at the initial time (t_0), the spore suspensions were treated in a water bath at 70°C for 5 min using the same capillary tube method.

Recovery. Volumes of 100 μl of the appropriate decimal dilutions of heat-treated spores were spread on brain heart agar (BHA; Biokar Diagnostics, Beauvais, France) at pH values ranging from 4.5 to 9.5 or incubated at temperatures ranging from 4°C to 40°C for *B. weihenstephanensis* and from 15°C to 60°C for *B. licheniformis*. BHA at a range of pH values was obtained as follows. BHI broth ($2\times$) was prepared and adjusted by addition of 1 M HCl to the desired pH, measured with a PHM210 pH meter (Meterlab, Villeurbanne, France) and a Tuff Tip electrode (Fisher Bioblock Scientific, Illkirch-Graffenstaden, France) previously calibrated using pH 4.00, pH 7.00, and pH 10.00 standard solutions. Then, the $2\times$ BHI broth was filtered on a 0.2- μm filter (Steritop system; Millipore Cor-

poration, Billerica, MA) and mixed with an equal volume of $2\times$ molten agar (30 g liter $^{-1}$). After the BHI broth and the agar had been mixed, the pH of the solidified and cooled medium was checked using the Tuff Tip electrode introduced into the top 1-cm layer of the agar. This pH value was recorded as the recovery pH for further experiments. Inactivation at a range of temperatures and recovery at optimum or suboptimal growth temperatures (8°C , 30°C , and 37°C for *B. weihenstephanensis* and 18°C , 45°C , and 58°C for *B. licheniformis*) and pH values (pH 5.20, 7.40, and 8.00 for both strains) were performed in triplicate, each replicate being performed with an independently prepared spore suspension. The full experimental design is presented in Table S1 in the supplemental material. Colony counts were recorded when they remained unchanged despite increasing incubation time. To ensure that BHA was sufficient for full spore germination, recovery after heat treatment on BHA supplemented with a 25 mM L-alanine–inosine mix triggering germination in *Bacillus* sp. strains (12, 13) or with 12.5 mg liter $^{-1}$ lysozyme was evaluated with spores of both strains, at optimal and one suboptimal temperature. Dehydration of recovery agar was monitored by weighing petri dishes for 20 days at 45°C . In this extreme condition, water loss was about 15% of the agar, resulting in a similar increase in the nutrient concentrations. Under most conditions tested, the incubation time was shorter and/or the temperature was lower. The medium dehydration effects on recovery were therefore assumed to be minor. Spores of both strains remained phase bright during incubation at room temperature for 15 min, which exceeds the time necessary for inclusion in molten agar and incubation at the target temperatures. Germination between the end of the heat treatment and incubation under test conditions was therefore considered negligible.

To ensure that spores density did not impact the recovery ability under our conditions, petri dishes of different sizes (4.5-cm, 9.0-cm, and 15-cm diameters) were inoculated with suspensions of heat-treated spores at 10^6 spores ml $^{-1}$, 10^7 spores ml $^{-1}$, and 10^8 spores ml $^{-1}$, similar to a previous work evaluating the influence of spore density on *Clostridium botulinum* germination (14). For each condition, the spores were incubated at 30°C and 8°C for *B. weihenstephanensis* and 45°C and 20°C for *B. licheniformis*. Under our conditions, there was no significant effect of spore density on recovery ability (data not shown).

Modeling. (i) Primary model. Heat inactivation curves were fitted with the model presented in equation 1 (15):

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

where N is the surviving population, N_0 is the initial spore population, δ is the time to the first decimal reduction, and p is a shape parameter. $\log(N)$ designates the decimal logarithm of N in this paper.

(ii) Secondary recovery model. The developed recovery model is derived from the gamma concept (22, 38) (equation 2):

$$\frac{1}{\delta_{(T_{\text{HT}})@ (T', \text{pH}')}} = \frac{1}{\delta_{\text{max}}^*} \lambda_{T_{\text{HT}}}(T_{\text{HT}}) \lambda_{T'}(T') \lambda_{\text{pH}'}(\text{pH}') \quad (2)$$

where $\delta_{(T_{\text{HT}})@ (T', \text{pH}')}$ is the apparent heat resistance of spores heat treated at temperature T_{HT} and then recovered at incubation temperature T' in agar medium at pH'; δ_{max}^* is the time to the first decimal reduction observed for the heat treatment temperature T^* and at the optimal recovery temperature (T'_{opt}) and the optimal recovery pH (pH'_{opt}); $\lambda_{T'}(T')$ and $\lambda_{\text{pH}'}(\text{pH}')$ describe the effect of incubation temperature T' and pH pH' during recovery (equations 3 and 4, respectively), and $\lambda_{T_{\text{HT}}}(T_{\text{HT}})$ is the lethal rate (equation 5) (16, 17). At the optimal recovery temperature and pH, $\lambda_{T'}(T')$ and $\lambda_{\text{pH}'}(\text{pH}')$ are equal to 1. At the reference temperature and pH, $\lambda_{T_{\text{HT}}}(T_{\text{HT}})$ is equal to 1. When recovery conditions become adverse, $\lambda_{T'}(T')$ and $\lambda_{\text{pH}'}(\text{pH}')$ increase and tend toward the infinite when T' and pH' become close to the limit of their domain of definition. The $\lambda_{T'}(T')$ and $\lambda_{\text{pH}'}(\text{pH}')$ functions were derived from the inverted Rosso function (18), where growth limits or cardinal values are input parameters (equation 2). Equations 3 and 4 for $\lambda_{T'}(T')$ and $\lambda_{\text{pH}'}(\text{pH}')$ are as follows:

$$\lambda_{T'}(T') = 1 / \frac{(T' - T'_{\max})(T' - T'_{\min})^{0.1}}{(T'_{\text{opt}} - T'_{\min})^{-0.9}[(T'_{\text{opt}} - T'_{\min})(T' - T'_{\text{opt}}) - (T'_{\text{opt}} - T'_{\max})(-0.9T'_{\text{opt}} + T'_{\min} - 0.1T')]} \quad (3)$$

for the recovery temperature range and

$$\lambda_{pH'}(pH') = 1 / \frac{(pH' - pH'_{\max})(pH' - pH'_{\min})^2}{(pH'_{\text{opt}} - pH'_{\min})[(pH'_{\text{opt}} - pH'_{\min})(pH' - pH'_{\text{opt}}) - (pH'_{\text{opt}} - pH'_{\max})(pH'_{\text{opt}} + pH'_{\min} - 2pH')]} \quad (4)$$

for the recovery pH range, where $T'_{\min}$, T'_{opt} , $T'_{\max}$, $pH'_{\min}$, pH'_{opt} and $pH'_{\max}$ are the minimal (min), optimal (opt), maximal (max) conditions of temperature or pH for recovery.

The effect of temperature used for the heat treatment was quantified by the Bigelow model (equation 5) (19):

$$\lambda_{T_{\text{HT}}}(T_{\text{HT}}) = 10^{\frac{T_{\text{HT}}^* - T_{\text{HT}}}{z_T}} \quad (5)$$

where T_{HT} is the heat treatment temperature and T_{HT}^* is the reference heat treatment temperature. T_{HT}^* was fixed at 90°C for *B. weihenstephanensis* and at 100°C for *B. licheniformis*. Finally, z_T is heat sensitivity, i.e., the change in heat treatment temperature leading to a 10-fold reduction of the decimal reduction time.

The model was fitted on the observations [$\log(\delta)$ or $\log(N)$] by minimizing the sum of squared errors (SSE) using lsqcurvefit function in MatlabR2012b (MathWorks, Natick, MA, USA). The goodness of fit of the model was checked by the corrected Akaike information criterion (AICc) and the RMSE (root mean square error) (20, 21). The smaller the AICc and the RMSE were, the better the model was fitted on the data. The 95% confidence intervals were calculated using the nlparci function in MatlabR2012b. The fitting performance of the model was statistically evaluated by the F test, comparing the mean square error of the model to the mean square error of the data. The computed f value was compared to the F table value (0.05 significance level). If the f value was lower than the F value from the table, the F test was accepted, indicating that the model fitting was statistically acceptable.

The fitting of models was compared with a likelihood ratio test and a test statistic, S_L , computed as follows:

$$S_L = n \log \text{RSS}_C - n \log \text{RSS}_U \quad (6)$$

where n is the number of data, RSS_C is the residual square sum for the constrained (C) model, and RSS_U is the residual square sum for the unconstrained (U) model. In this work the constrained models were those (i) using a unique p value (equation 1) or a unique z_T value (equation 5) or (ii) using predetermined cardinal temperatures and pH for each strain (equations 3 and 4). The unconstrained models were those (i) using one p value for each inactivation curve or one z_T value for each of the $\log(\delta) = f(T')$ and $\log(\delta) = f(pH')$ curves or (ii) using the cardinal parameters estimated by the model. S_L is low when the selection of the model has no significant incidence on the quality of fitting. When n tends toward infinity, the limiting distribution of S_L is χ^2 distributed with $p_U - p_C$ degrees of freedom, where p_U is the number of parameters in the unconstrained model and p_C is the number of parameters in the constrained model. If S_L is lower than χ^2 ($\alpha = 0.05$), the difference in the fitting of both models is considered not significant.

RESULTS

A total of 115 inactivation curves for *B. weihenstephanensis* spores and 78 for *B. licheniformis* spores were performed, each with at least six counts of survivors and 4 log reductions (see Tables S1 to S5 in the supplemental material). The inactivation curves (Fig. 1) were fitted with equation 1 (22). No change in the inactivation curve shape (p value) was noted according to sporulation, heat treatment, and recovery conditions. Instead of a different p value

FIG 1 Inactivation curves of *B. weihenstephanensis* spores heat treated at 90°C and incubated at pH 5.20 (▲) and pH 7.40 (●) at 30°C. The lines correspond to the fitting of the data with equation 1 (22).

for each inactivation curve, a single p value was estimated for all inactivation curves. This p value was estimated at 0.68 ± 0.03 for *B. weihenstephanensis* and 1.96 ± 0.13 for *B. licheniformis*, as the curve shapes were concave upward and concave downward, respectively. No significant difference between the p values estimated for each inactivation curve and the p value estimated for all the inactivation curves was detected (likelihood ratio test, $\alpha = 0.05$).

The impact of recovery temperature was significant only at temperatures close to the recovery boundaries. The extreme temperatures at which recovery was observed were 6°C and 39°C for *B. weihenstephanensis* and 17°C and 59°C for *B. licheniformis*. For *B. weihenstephanensis*, the mean log values of δ , the apparent heat resistance, were $\log 0.31 \pm 0.06$ min at 8°C, $\log 0.39 \pm 0.07$ min at 30°C, and $\log 0.39 \pm 0.07$ min at 37°C; for *B. licheniformis*, the mean log(δ) values were $\log 0.98 \pm 0.06$ min at 18°C, $\log 1.14 \pm 0.01$ min at 45°C, and $\log 1.00 \pm 0.06$ min at 58°C, for the reference heat treatment temperature. No significant changes in the δ value were observed from 8°C to 37°C for *B. weihenstephanensis* and from 18°C to 58°C for *B. licheniformis* (analysis of variance [ANOVA], $\alpha = 0.05$). The time required for survivors to form a colony was unsurprisingly greater at suboptimal temperatures than at optimal growth temperatures. For example, the time to colony counting for *B. weihenstephanensis* KBAB4 was 24 h at 30°C and pH 7.40 and 20 days at 7°C and pH 7.40.

There was a progressive decrease in δ values as the recovery pH came close to the pH recovery limits. The estimated optimal recovery pH was 7.80 ± 0.23 for *B. weihenstephanensis* and 7.73 ± 0.13 for *B. licheniformis*. A decrease in the recovery pH from 7.00 to pH 5.50 caused a 3-fold decrease in δ values of both *B. weihenstephanensis* and *B. licheniformis*. Neither strain formed any colonies at a recovery pH lower than 4.70. The δ value replicates for each strain was determined at the optimal recovery pH and at two suboptimal recovery pHs. For *B. weihenstephanensis*, the mean log(δ) values were $\log -0.04 \pm 0.10$ min at pH 5.40, $\log 0.34 \pm 0.13$ min at pH 7.40, and $\log 0.37 \pm 0.11$ min at pH 8.00; for *B. licheniformis*, the mean log(δ) values were $\log 0.41 \pm 0.07$ min at pH 5.40, $\log 1.09 \pm 0.03$ min at pH 7.40, and $\log 0.96 \pm 0.21$ min at pH 8.00. Similarly, spore recovery was slower at suboptimal pH than at optimal pH. For example, the time to counting for *B. weihenstephanensis* was 24 h at pH 7.40 and at 30°C and 15 days at pH 5.10 and 30°C.

FIG 2 Effect of recovery temperature and pH on spores of *B. weihenstephanensis* (A and C) produced at 30°C (optimal temperature) (solid symbols) and 12°C (suboptimal temperature) (open symbols) and treated at 95°C (squares), 90°C (circles), and 85°C (diamonds) and on spores of *B. licheniformis* (B and D) produced at 45°C (optimal temperature) (solid symbols) and 20°C (suboptimal temperature) (open symbols) and treated at 105°C (squares), 100°C (circles), and 95°C (diamonds) on apparent heat resistance [$\log(\delta)$]. The vertical dashed lines represent the boundaries of temperature or pH beyond which no recovery was observed. The full lines correspond to the fitting of the data to the model developed in the present work (see Materials and Methods for details).

The spores produced at a suboptimal sporulation temperature behaved similarly to those formed at the optimal sporulation temperature (i.e., they showed same trend in terms of the influence of recovery temperature and pH). The major difference was in the $\delta_{\max}$ value, which was lower at suboptimal sporulation temperature (Fig. 2). The spores produced under optimal conditions were treated at three different temperatures in order to estimate their heat sensitivity (z_T value). From each recovery condition, it was possible to estimate a z value. Whatever the recovery temperature and pH, the z value was between 7.3°C and 8.8°C for *B. weihenstephanensis* (19 z values) and between 7.0°C and 8.0°C for *B. licheniformis* (10 z values). No significant difference could be discerned between each z value estimated on each recovery conditions and the single estimated z value (likelihood ratio test, $\alpha = 0.05$). The heat sensitivity (z value) was therefore assumed to be constant in the range of tested conditions for both strains and equal to $8.02^\circ\text{C} \pm 0.26^\circ\text{C}$ for *B. weihenstephanensis* and $7.67^\circ\text{C} \pm 0.27^\circ\text{C}$ for *B. licheniformis*. The heat treatment temperature has an impact only on spore heat resistance (Fig. 2). There was no interaction between heat treatment temperature and recovery conditions: the z_T value was not impacted by the recovery conditions.

Modeling the effect of recovery temperature and pH conditions on the spores' heat resistance. Equation 2 was used to model the spores' apparent heat resistance according to the heat treatment temperature and the recovery temperature and pH. The z value, corresponding to heat sensitivity, was considered constant for both strains (see above). The model was fitted on 115 $\log(\delta)$

values for *B. weihenstephanensis* and on 78 $\log(\delta)$ values for *B. licheniformis*. $T'_{\min}$, T'_{opt} , $T'_{\max}$, $\text{pH}'_{\min}$, pH'_{opt} , and $\text{pH}'_{\max}$ were estimated for each strain. The RMSE values were 0.15 and 0.11 for *B. weihenstephanensis* and *B. licheniformis*, respectively (Table 1). These RMSE values are low compared to the standard deviation of $\log(\delta)$ values from replicated inactivation curves. Moreover, the fitting performance of the model was statistically accepted by the F test (with an α value of 0.05). Consequently, the model derived from equation 2 satisfactorily describes the recovery behavior of heat-treated spores of both strains.

Recovery after heat treatment is linked to incubation temperature and pH. Temperature and pH growth limits are among the most commonly available characteristics of bacteria. One option of the model was to use previously published growth limits (23), and therefore to fix the recovery parameters. No significant difference could be detected between the fitting with all estimated parameters and the fitting with fixed cardinal values for both strains (likelihood ratio test, $\alpha = 0.05$).

DISCUSSION

The spore heat resistance of many *Bacillus* sp. is highly impacted by the sporulation temperature (2). As shown in previous work (10), the sporulation temperature has an impact mainly on spore heat resistance (expressed with δ values in this work) but does not impact heat sensitivity (expressed with z_T values in this work). The specific effect of recovery conditions is the same whatever the sporulation and heat treatment conditions. Heat treatment leads

TABLE 1 Cardinal recovery parameters and growth cardinal values for *B. weihenstephanensis* KBAB4 and *B. licheniformis* Ad978 estimated with fixed growth parameters and with estimation of all parameters

Model fitting	Parameter	Estimated value (confidence interval ^c) for strain	
		<i>B. weihenstephanensis</i> KBAB4	<i>B. licheniformis</i> Ad978
Temp and pH growth limits as fixed parameters	Estimated heat treatment parameters		
	Log(δ^*_{opt}) (log min) ^a	0.36 (0.34; 0.38)	1.10 (1.08; 1.12)
	Log(δ^*_{opt}) (log min) ^b	−0.07 (−0.10; −0.04)	0.15 (0.12; 0.18)
	z_T (°C)	8.05 (7.79; 8.31)	7.66 (7.32; 8.00)
	Predetermined cardinal temp and pH ^d		
	T_{min} (°C)	2.72 (0.38; 5.60)	11.30 (6.12; 17.66)
	T_{opt} (°C)	31.91 (30.93; 32.60)	49.01 (47.52; 50.34)
	T_{max} (°C)	40.91 (40.41; 41.84)	57.87 (56.27; 65.83)
	pH _{min}	4.35 (4.16; 4.51)	4.63 (4.43; 4.85)
	pH _{opt}	7.71 (7.55; 7.95)	8.17 (7.86; 8.72)
	No. of data	115	78
	RMSE	0.15	0.14
Heat treatment parameters and recovery limits as estimated parameters	Estimated heat treatment parameters		
	Log(δ^*_{opt}) (log min) ^a	0.38 (0.34; 0.42)	1.11 (1.08; 1.14)
	Log(δ^*_{opt}) (log min) ^b	−0.04 (−0.09; −0.01)	0.16 (0.12; 0.20)
	z_T (°C)	8.06 (7.80; 8.32)	7.67 (7.40; 7.94)
	Estimated recovery limits		
	T'_{min} (°C)	5.94 (5.82; 6.06)	16.76 (15.79; 17.73)
	T'_{opt} (°C)	36.37 (24.63; 48.12)	31.79 (28.40; 35.18)
	T'_{max} (°C)	38.03 (37.63; 38.42)	65.97 (56.94; 75.01)
	pH' _{min}	3.79 (3.01; 4.58)	4.54 (4.41; 4.68)
	pH' _{opt}	7.80 (7.53; 8.07)	7.73 (7.61; 7.86)
	pH' _{max}	10.34 (9.93; 10.75)	9.80 (9.69; 9.90)
	No. of data	115	78
	RMSE	0.15	0.11

^a Log(δ^*_{opt}), optimal heat resistance, at the reference temperature, for spores produced at the optimal temperature.
^b Optimal heat resistance, at the reference temperature, for spores produced at the suboptimal temperature.
^c $\alpha = 0.05$.
^d Data are from reference 23.

to the inactivation of spores, but some can be sublethally injured and are able to germinate, multiply, and form a colony (24). Supplementation of the recovery medium with an alanine-inosine mix, which is known to trigger germination on *B. weihenstephanensis* and *B. licheniformis* (12, 13), or with lysozyme, which is known to restore the germination of damaged spores (8), had no effect on recovery (i.e., similar counts were obtained after heat treatment on recovery agar, supplemented or not). Consequently the observed effect is likely due to impaired germination subsequent to damage and also to a reduced ability of the germinated cells to adapt to suboptimal temperature conditions to form colonies. The developed model satisfactorily describes the recovery behavior of heat-treated *Bacillus* sp. spores, accounting for pre-treatment, per-treatment, and posttreatment conditions. The range of pH and temperature allowing the recovery of spores of the tested *B. weihenstephanensis* and *B. licheniformis* strains was within the range of temperature and pH allowing growth. The domain of growth temperatures and the domain of recovery temperatures have very close boundaries. Consequently, the current model used predetermined cardinal temperature and pH values for each strain as control parameters, because these values have a real biological meaning and are reliable estimators of growth lim-

its (25). The cardinal temperatures T_{min} and T_{max} are, respectively, the temperature below which and the temperature above which growth cannot theoretically be observed (26). The minimal temperature for growth estimated by a cardinal temperature model (T_{min}) is always a few degrees Celsius lower than the observed minimum temperature allowing growth (27). As we demonstrated, these values can be used as input parameters to estimate the apparent heat resistance at given recovery temperatures and pH values. The recovery behavior of bacterial spores after heat treatment can therefore be modeled with parameters that have a biological meaning and that are relatively easily accessible to the scientific community, for instance, through literature review.

The impact of recovery temperature on the spore colony-forming ability is low in the recovery range. This has also been observed for different species, such as *Bacillus cereus* CNRZ 110, *Alicyclobacillus acidoterrestris* ATCC 49025, and several strains of *Bacillus stearothermophilus* (4, 28, 39). Only the time taken to form a colony was significantly influenced by the recovery temperature. Recently, a model describing the effect of different factors on the lag time of *B. cereus* spores has been developed (29). In this study, the observed biological response is the estimated lag time corresponding to the time taken for spores to germinate, outgrow, and

grow, taking into account only the time required to detect the germination and growth of at least one spore. The effect of recovery temperature could be explained by a prolongation of the germination and outgrowth duration as the temperature approaches the growth boundaries, as demonstrated for several *Bacillus* and *Clostridium* species (30, 31, 33, 40), and by the decrease in growth rates at temperatures lower or higher than the optimal temperature. Many foods, such as refrigerated ready-to-eat foods or cooked chilled foods, are processed with mild heat treatments and rely on refrigeration for preservation and/or combining suboptimal pH with low temperature as additional hurdles to prevent growth of surviving pathogenic or spoilage spore-forming bacteria (32). Our results suggest that in these foods, storage at low temperature will mainly delay the growth of spore-forming bacteria, not prevent the growth of surviving spores, and that recovery pH could actually affect the recovery ability of surviving spores. The recovery pH has a more progressive effect on the colony-forming ability, mathematically described with an exponent value of 2.0 in equation 4. pH values near the optimal growth pH offer the highest colony formation ability for both strains. Germination rates at low pH values may be lower and/or colony formation slower, as previously observed for *B. cereus*, for instance (41, 42), and *C. botulinum* (34). As with temperature, the domain of growth pH values and the domain of recovery pH values have very close boundaries. Prolonged outgrowth caused by low pH could be due to the H^+ effect on cytoplasmic pH and, although this would be highly strain/species dependent, to significant inner pH modifications during germination (35). A slight change of temperature or pH near to the boundaries caused a dramatic decrease in apparent heat resistance values. This can be explained by the growth behavior of bacterial cells under conditions close to the growth/no-growth boundaries, where the probability of cells forming a colony is lower than that under optimal conditions (36). This phenomenon could be strengthened by a decrease in the probability of germination of surviving spores. Moreover, an effect of spore density on spore germination has been shown, with *Clostridium* sp., for instance (14). Again, the phenomenon is complicated by the release of dipicolinic acid, triggering germination, during spore germination.

Interestingly, the number of inactivated cells can be described by cumulating the heat inactivation effect and the inhibitory effect due to suboptimal recovery conditions (equation 7).

$$n = n_{HT} + n' \quad (7)$$

where n is the apparent total log reduction, n_{HT} is the log reduction due to heat treatment, and n' is the virtual decimal decrease due to recovery conditions.

Equation 1 can therefore be written as follows:

$$n = \log \frac{N_0}{N} = \left(\frac{t}{\frac{\delta_{max}}{\lambda_{HT} \lambda_T \lambda_{pH}}} \right)^p \quad (8)$$

This equation is equivalent to equation 9:

$$n = \left(\frac{1}{\delta_{max}} t \cdot \lambda_{HT} \right)^p \cdot (\lambda_X')^p \quad (9)$$

where the effect of heat treatment could be expressed by

$$n_{HT} = \left(\frac{1}{\delta_{max}} \cdot t \cdot \lambda_{HT} \right)^p \quad (10)$$

and the effect of recovery by

$$n' = n_{HT} [(\lambda_X')^p - 1] \quad (11)$$

The impact of recovery can be calculated knowing the impact of the heat treatment (n_{HT}) and of the recovery medium formulation $[(\lambda_X')^p]$. There is no effect of the recovery environment ($n' = 0$) when λ_X' is equal to 1, i.e., when the recovery conditions are optimal. On the contrary, when the recovery conditions are beyond the recovery limits [λ_X' tends to $+\infty$], colony formation on the recovery medium is fully inhibited (n' tends to $+\infty$). This can also be linked to the germination rate, where the influence of heat treatment intensity and recovery temperature and pH are taken into account.

In conclusion, using a proper set of parameters for each strain and a model based on generic mathematical functions, the recovery of *B. weihenstephanensis* and *B. licheniformis* spores after heat treatments at diverse temperatures and as a function of the incubation temperature and the pH of the recovery medium was quantified. A similar approach can be used to quantify the impact of pH in addition to temperature during heat treatment on the recovery of spores. In this new model, only the heat resistance at optimal recovery temperature and pH has to be estimated, since the other parameters—cardinal growth temperature and pH—are obtained from independent experiments/sources. The spore population considered here is the population able to germinate and recover physiological activity in order to form a colony on nutrient agar. It remains undetermined whether the germination or the adaptation of the germinated cell is affected by sublethal heat treatment. The biological process leading to the formation of a colony from a stressed spore is likely stochastic, and further research is needed to quantify the relative part of these two steps in the spore recovery process.

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