Food Microbiology 81 (2019) 115–120

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Quantifying permeabilization and activity recovery of *Bacillus* spores in adverse conditions for growth

C. Trunet[a,*], H. Ngo[a], L. Coroller[b]

[a] Université de Brest, EA3882, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, UMT14.01 SPORE-RISK, ISBAM, Institut Universitaire et Technique, 6 rue de l'Université, F-29334 Quimper, France

[b] Université de Brest, EA3882, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, UMT14.01 SPORE-RISK, ISBAM, Ecole Supérieure d'Ingénieur en Agroalimentaire de Bretagne Atlantique, 6 rue de l'Université, F-29334 Quimper, France

ARTICLE INFO

Keywords:
Germination
Spore forming bacteria
Flow cytometry
Predictive microbiology

ABSTRACT

Heat treatment is the main hurdle used to eliminate spores in foods but the pH conditions which spores encounter after the treatment have a tremendous impact on the spore ability to germinate, outgrow and grow. The aim of this work was to discriminate the inactive permeable spores and the active spores in unfavorable acidic conditions, after a heat treatment. In this study, *Bacillus weihenstephanensis* KBAB4 was used as model microorganism for psychrotrophic *Bacillus*. The spores were heat treated to inactivate 90% of the population, 12 min at 85 °C, or 2 min at 95 °C. After each treatment the spores were incubated at pH 5.50 or pH 7.40. The evolution of dormant spores, permeable spores, germinated and vegetative cells was monitored by flow cytometry using a double staining. LDS 751, stains in red all the permeable cells, and CFDA stains in green cells presenting an esterase activity. Dormant spores did not show neither red fluorescence nor green fluorescence. Permeabilized spores which did not recover metabolic activity were red fluorescent but not green fluorescent. Germinated spores (permeabilized and having an esterase activity) appeared red fluorescent and green fluorescent due to their permeability and their metabolic activity. This method allowed the differentiation of the impact of heat treatment and post-treatment incubation pH on the two first steps of germination: spore permeabilization and activity recovery. Having a better understanding of spore germination at unfavorable post-treatment pH allows a better control of spore forming bacteria in foods.

1. Introduction

Bacillus spores can resist heat treatment which is the main hurdle used in food industry to preserve food stability. The survival spores are able to germinate, outgrow and multiply in products leading to poisoning or spoilage issues (Abee et al., 2011). Quantifying the potential of germination and outgrowth of bacterial spores in adverse conditions is challenging. Indeed, spore capacity to germinate and outgrow are impacted by sporulation conditions, heat treatment intensity and incubation conditions (Mtimet et al., 2015; Trunet et al., 2015). From germination to multiplication *Bacillus* spores pass through successive steps. The germination consists in a first phase of partial rehydration of the core followed by the lysis of the cortex peptidoglycan by dedicated enzyme (Cortex Lytic enzymes). Then, the spore core is rehydrated, swells up and outgrows from the outer layers of the spore. It is generally considered that metabolic activity is recovered during the outgrowth process (Setlow, 2014, 2013). Different methods can be used to observe these different stages (Trunet et al., 2017). A widespread method used to observe spore germination is the phase contrast microscopy. Indeed, dormant spores are highly dehydrated and appear phase bright using contrast microscopy. Germinated spores are distinguished from dormant spores thanks to the loss of the refractility observed when the spores are rehydrated during germination (Pandey et al., 2013). Using epifluorescence microscopy or flow cytometry, germinated spores are discriminated from dormant spores thanks to their permeability to fluorescent markers, such as Syto9 (van Melis et al., 2014, 2011). But the permeabilization step of spores do not inevitably lead to outgrowth (Mtimet et al., 2017; Wang et al., 2011). The proportion of spores able to recover after permeabilization can be modulated by process such as heat treatment, high pressure as well as the conditions encountered after the treatment (Mathys et al., 2007; Mtimet et al., 2017).

The aim of this work was to discriminate the permeabilization step from the activity recovery step of heat treated spores exposed to unfavorable conditions for bacterial growth. Using flow cytometry, spore permeability can be monitored using staining with small molecules, such as Syto9, and metabolic activity can be observed via, for example,

* Corresponding author.
E-mail address: clement.trunet@univ-brest.fr (C. Trunet).

https://doi.org/10.1016/j.fm.2018.06.013
Received 30 November 2017; Received in revised form 15 June 2018; Accepted 21 June 2018
Available online 22 June 2018
0740-0020/

respiratory activity (CTC staining) or esterase activity (CFDA). *Bacillus weihenstephanensis* KBAB4 was studied in this work as a model for *B. cereus*. It needs to achieve germination, outgrowth and the recovery of metabolic activity to produce toxins. In the same way, spoiling sporeforming bacteria must achieve the same process leading to the production of enzyme and finally to the spoilage of the food.

2. Material and methods

2.1. Bacterial strain and cultures

The *Bacillus weihenstephanensis* strain KBAB4 (INRA, Avignon, France) was stored at −80 °C in 1.5 mL aliquot Eppendorf tubes of Brain Heart Infusion (BHI, Biokar Diagnostics, Beauvais, France) mixed with 50% glycerol (vol.vol^{-1}). A flask of BHI (100 mL) was inoculated with 1 mL of the stock suspension and incubated for 8 h at 30 °C, then 1 mL was transferred into 100 mL of BHI for 16 h at 30 °C. Finally, 0.1 mL of *B. weihenstephanensis* suspension was added to 100 mL BHI and was incubated for 6 h at 30 °C. The final cell concentration in the culture was approximately 10^8 CFU.mL^{-1}. The number of spores estimated by the number of cells surviving a heat treatment of 5 min at 70 °C was lower than 100 spore CFU.mL^{-1}. This suspension was used for spore preparation.

2.2. Spore preparation

Spores were produced through a two-step sporulation process (Baril et al., 2011). A flask of 100 mL of the previously described culture were centrifuged (6000 x *g*, 10 min, 12 °C) and the vegetative cells were resuspended in 100 mL of sporulation mineral buffer (SMB) at pH 7.00 (4.5 g.L^{-1}K_2HPO_4 at, 1.8 g.L^{-1}KH_2PO_4 at, 8.0 mg.L^{-1} $CaCl_2$, H_2O and 1.5 mg.L^{-1}$MnSO_4$, H_2O) or in 100 mL of SMB at pH 5.50 (K_2HPO_4 at 5.04 g.L^{-1}, KH_2PO_4 at 0.35 g.L^{-1}, $CaCl_2$, H_2O at 8.0 mg.L^{-1} and $MnSO_4$, H_2O at 1.5 mg.L^{-1}), filter-sterilized using 0.2 µm pore size filters (Baril et al., 2011). The cell suspensions in SMB at pH 7.00 were incubated at 30 °C, 100 rpm. Spores in SMB were harvested when free spores represented more than 95% of cells under X1000 magnification in phase-contrast microscopy (Olympus BX50, Olympus Optical Co., Ltd, Hamburg, Germany), *i.e.* after 1–2 days of incubation at 30 °C. The spore suspensions were centrifuged (6000 x *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 aliquot volumes and stored for 1 month at 4 °C before use. The final concentrations of the stock suspensions were 10^8 spores.mL^{-1} for *B. weihenstephanensis* cultures in SMB at 30 °C and pH 7.00 (Trunet et al., 2015).

2.3. Heat treatment

The spores of *Bacillus weihenstephanensis* KBAB4 were diluted in PBS to a final concentration of around 10^8 spores/mL. Capillary tubes of 200 µL were filled with 100 µL of spore suspension and sealed, then immersed into a water/glycerol bath maintained at 85 °C during 12 min or at 95 °C during 2 min (Baril et al., 2011; Trunet et al., 2015) in order to obtain the inactivation of 90% ± 0.2 of the initial spore population when spores were incubated at pH 7.40, 30 °C after heat treatment, and an inactivation of 96% ± 0.2% when spores were incubated at pH 5.50, 30 °C after heat treatment.

In order to verify the heat treatment efficiency at 85 °C or 95 °C, capillary tubes were removed from the heat treatment bath at appropriate times 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). Volumes of 1 mL of the appropriate decimal dilutions of heat-treated spores were mixed into molten Brain Heart Agar (BHA, Biokar Diagnostics, Beauvais), and incubated at 30 °C. To estimate the spore concentration at the initial time (t_0), the microbial suspensions were treated in a water bath at 70 °C for 5 min using the same capillary tube method.

2.4. Evolution of heat-treated spores in different pH conditions

Control samples of dormant spores, inactive permeable spores, active permeable spores and vegetative cells were analyzed on a flow cytometer (Cyflow space, Sysmex Europe Gmbh, Norderstedt, Germany) equipped with an excitement light source at 488 nm laser (50 mW) and four detectors: FSC (Forward Scatter), SSC (Side Scatter) (detecting light emission at 488 nm), Fluorescence Light detector with a 536/40 nm filter (FL1) and Fluorescence Light detector with a 610 nm high-pass filter (FL2). The software used to collect and analyze the flow cytometry data was *Flomax 2.3* (Cyflow space, Sysmex Europe Gmbh, Norderstedt, Germany). The control sample for dormant spores was obtained by the suspension of spores stored at 4 °C in sterile distilled water. The control sample for inactive permeable spores was obtained by incubating dormant spores in sterile distilled water with 25 mM L-alanine and 12.5 mM inosine during only 10 min at 30 °C. The active permeable spore control sample was obtained by incubating dormant spores in BHI during 20 min at 30 °C. The vegetative cell control sample was obtained incubating dormant spores in BHI during 2 h at 30 °C.

The spores and vegetative cell could be distinguished by their size, corresponding to FSC values, and their granularity corresponding to SSC. Spores and vegetative cells were stained with 5-Carboxyfluorescein Diacetate (CFDA, Molecular Probes, Life Technologies, Saint Aubin, France), a green fluorescent marker of esterase activity. Spores and vegetative cells were also stained with Laser Dyes Styryl 8 (LDS751 Molecular Probes, Life Technologies, Saint Aubin, France), a red fluorescent cell-permeant DNA intercalate allowing the staining of all spores and cells excepting dormant spores.

The observation by flow cytometry was completed with phase contrast microscopy and epifluorescence microscopy observations. The spores were confirmed to be dormant as they appeared as phase bright spores and no fluorescent. The permeable spores were confirmed as they appeared phase dark and red fluorescence. The germinated spores were confirmed as they appeared red and green fluorescent, as well as vegetative cells.

The regions were confirmed analyzing mixes of refractive spores and permeable spores; refractive spores and germinated spores; permeable spores and germinated spores; refractive spores, permeable, germinated spores and vegetative cells.

2.5. Germination, outgrowth and growth recovery kinetics by flow cytometry

A volume of 10 mL of BHI was inoculated at 10^6 spores.mL^{-1} and incubated. The BHI pH was 7.4 or was modified by 1M HCl addition to pH 5.5 and flasks were placed under agitation (110 rpm). A volume of 0.1 mL was sampled at regular times and diluted to 10^5 spores/cells.mL^{-1} in Phosphate Buffer Saline (PBS, K_2HPO_4 at 4.5 g.L^{-1} and KH_2PO_4 at 1.8 g.L^{-1}, NaCl at 8 g.L^{-1}, 0.22 µm filtered) and stained with LDS 751 at a final concentration of 0.5 mM and CFDA at 1 mM. The spores and/or cells were quantified in exactly 200 µL using the probe system of the flow cytometer (Cyflow space, Sysmex Europe Gmbh, Norderstedt, Germany).

At each time the numbers of dormant spores, permeable spores, germinated spores and vegetative cells were quantified in at least duplicates, each replication being performed with independently prepared spore suspension. For each test, the assays were stopped when the first cell divisions were observed, as the total cell number, determined by flow cytometry, significantly increased.

2.6. Quantification of each physiological stage among time

During germination and outgrowth kinetics, the cell population was hypothetically composed of dormant spores, permeable spores,

germinated spores and vegetative cells. The total cell number over time could be described as:

$$N_T(t) = N_R(t) + N_P(t) + N_A(t) + N_V(t) \quad (1)$$

where, N_T was the total number of bacterial cells, N_R was the number of refractive spores, N_P was the number of permeable spores, N_A was the number of active permeable spores, N_V was the number of vegetative cells and t was the time of incubation.

The germination and outgrowth process is a succession of four steps that could be distinguished by flow cytometry, *i.e.* the transformation from dormant spores to inactive permeable spores, from inactive permeable spores to active permeable spores and from active permeable spores to vegetative cells. Reverse transformation or direct transformation of dormant spores to vegetative cells was not demonstrated. Moreover, the total number of events $N_T(t)$ was stable during all the assays. The evolution of each population was described using mathematical models described afterwards.

The evolution curves of spore populations were fitted using the model inspired from Weibull distribution (equation (2)).

$$P_i(t) = 1 - 10^{\left(\frac{t}{t_i}\right)^{S_i}} \quad (2)$$

where, P_i corresponded to the Weibull distribution of time, t_i was the time needed to reach 90% of the maximal number of cells of population i (refractive spores, inactive permeable spores, active permeable spores or vegetative cells), S_i was a reflection the scattering of t_i within the population: the higher S_i was the lower the scattering was.

The decrease of refractive spore population could be described by the proportion of spores which were able to be permeabilized, within the initial population of refractive spores.

$$N_R(t) = N_{R_0} \cdot (1 - \tau_p \cdot P_p(t)) \quad (3)$$

where $N_R(t)$ was the refractive spore number, N_{R_0} was the initial number of refractive spores, τ_p was the maximal proportion of refractive spores able to be permeabilized and $P_p(t)$ was the distribution as described of the permeabilization time (equation (2)).

The permeable spore population was submitted to two phenomena. Firstly, the permeable spore population increased as refractive spores decreased and secondly, a proportion of permeable spores was able to recover a metabolic activity (then considered as germinated). The evolution curves of permeable spores were fitted using the model presented in equation (4).

$$N_P(t) = \left(N_{R_0} - N_R(t)\right) \cdot (1 - \tau_{act} P_{act}(t)) \quad (4)$$

where (N_p) was the permeable spore number, N_{R_0} was the number of refractive spores at the initial time, N_R was the number of refractive spores, τ_{act} was the proportion of permeable spores able to be germinate. As previously described, P_{act} was the distribution of activity recovery time (equation (2)).

The active permeable spore population increased from the permeabilization of spores. A proportion of the germinated spores was able to outgrow so the germinated spore population decreased. The evolution of the germinated spore population was fitted using the model presented in equation (5).

$$N_A(t) = \left(N_{R_0} - N_R(t)\right) \cdot (1 - \tau_{outgr} P_{outgr}(t)) \quad (5)$$

where (N_A) was the active permeable spore number, N_{R_0} was the number of refractive spores at the initial time, N_R was the number of refractive spores, τ_{outgr} was the proportion of germinated spores able to outgrow. As described for permeable spores, P_{outgr} was the distribution of outgrowth time (equation (2)).

The vegetative cells increased as the germinated spore population decreased, and were fitted using the model presented in equation (6).

$$N_V(t) = \left(N_{V_{max}}\right) \cdot (1 - \tau_{outgr} \cdot P_{outgr}(t)) \quad (6)$$

where (N_{VC}) was the vegetative cell number, $N_{V_{max}}$ was the maximal number of vegetative cells, τ_{outgr} was the proportion of germinated spores able to outgrow.

The four equations allowed the quantification of time and proportion of permeabilization, germination and outgrowth of heat treated or untreated spores, incubated in different conditions of pH.

2.7. Statistics

The model was fitted on the observations by minimizing the sum of squared errors (SSE) using *lsqcurvefit* function from *MatlabR2*012b (The Math-works, Natick, USA). The goodness of fit of the model was checked by the RMSE (Root Mean Square Error) (Huet et al., 2010; Scherrer et al., 2009). The smaller the RMSE were, the better the model was fitted the data. The 95% confidence intervals were calculated using the *nlparci* function from *MatlabR2*012b. 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. Moreover, variance comparison tests were performed using the *anova* and *anova2* functions from *MatlabR2*012b.

3. Results

The refractive spores, inactive permeable spores, active permeable spores and vegetative cells were distinguished thanks to their permeability and/or their metabolic activity. The dormant spores which are impermeable to many molecules were stained neither with LDS 751 nor CFDA. Mean value of green and red fluorescence for dormant spores were respectively 0.35 ± 0.14 a.u. (FL1), corresponding to no metabolic activity, and 0.31 ± 0.16 (FL2) corresponding to no permeability.

The spore population which was stained with LDS 751 and showed a low fluorescence with CFDA, meaning that they did not show esterase activity, was identified as the inactive permeable spore population. The mean fluorescence values for permeable spores were 0.37 ± 0.16 a.u. (FL1) and 4.08 ± 1.98 a.u. (FL2).The spores which were stained with LDS 751 and CFDA were identified as active permeable spores as they were permeable to LDS 751 and recovered an esterase activity. The mean fluorescence values for active permeable spores were 6.72 ± 3.7 a.u. (FL1) and 3.91 ± 0.27 a.u. (FL2). Finally, the vegetative cell population was identified thanks to their higher fluorescence intensity with LDS 751 and CFDA staining. Indeed, the mean fluorescence values for vegetative cells were 49.68 ± 27.6 a.u. (FL1) and 16.52 ± 7.07 a.u. (FL2). The populations described previously are shown in Fig. 1. The flow cytometry method we developed allowed the quantification of events belonging to each population regarding time.

A mathematical model was proposed in order to describe the evolution of each population as a function of time (Fig. 2). The model was based on Weibull model, which takes into account the individual variability within each studied population, as well as on a set of parameters describing the evolution of each population: the initial number of spores (N_0), the time needed to reach 90% of the inactive permeable spore population (t_p), the dispersion of individual permeabilization times (S_p), the time needed to reach 90% of the active permeable spore population (t_{act}), the dispersion of individual activity recovery times (S_{act}), the time needed to reach 90% of the vegetative cell population (t_{outgr}), the dispersion of individual outgrowth times (S_{outgr}). All parameters were estimated for each tested condition of heat treatment and incubation pH.

The control condition corresponded to untreated spores incubated at pH 7.4. Within the initial population, 95 ± 1% (mean value ± standard deviation) of spores was able to lose their permeability, τ_p, and the time of permeabilization, t_p, was 3.32 ± 0.55 min. Then 95 ± 1% of permeable spores were able to recover an esterase activity,

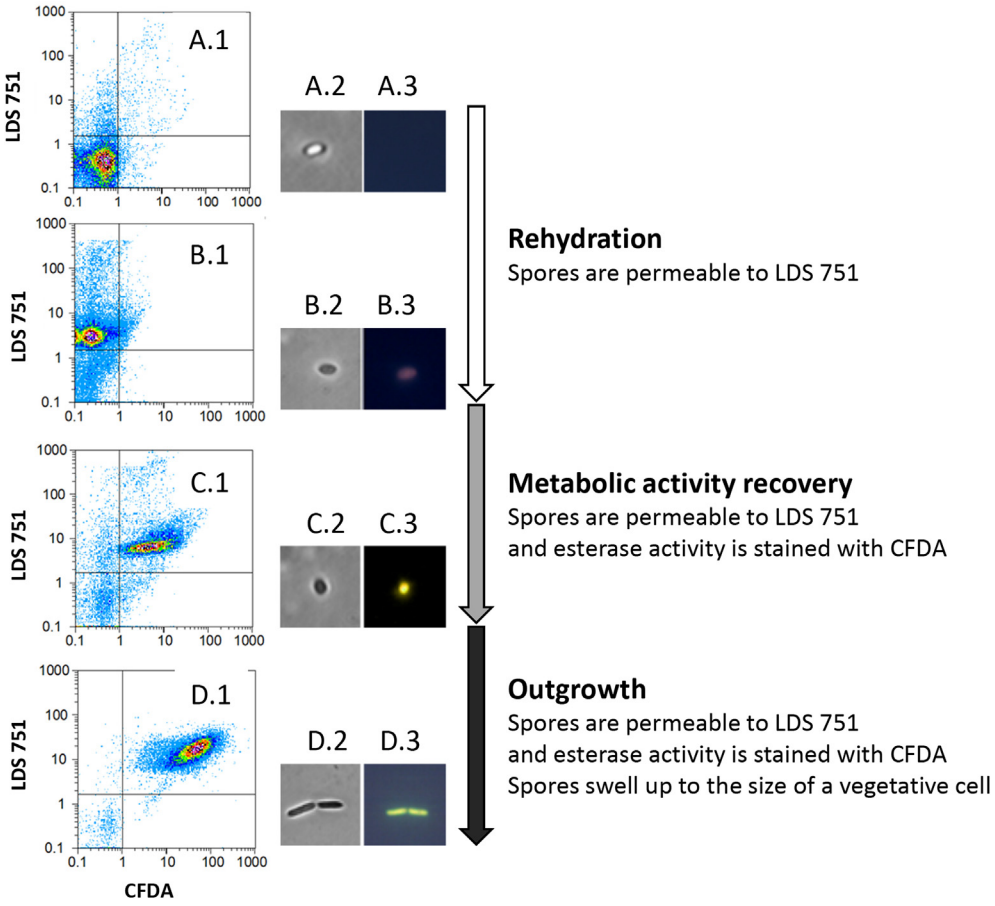

Fig. 1. Evolution of (A) dormant spores presenting a staining neither by LDS 751 nor CFDA by flow cytometry (A.1), bright phase state was verified by phase contrast microscopy (A.2) and fluorescence by epifluorescence microscopy (A.3); (B) inactive permeable spores presenting a staining with LDS 751 but not with CFDA (B.1), dark phase state was verified by phase contrast microscopy (B.2) and fluorescence by epifluorescence microscopy (B.3); (C) active permeable spores presenting a staining with LDS 751 and CFDA, dark phase state was verified by phase contrast microscopy (C.2) and fluorescence by epifluorescence microscopy (C.3); (D) vegetative cells presenting higher intensity staining with LDS 751 and CFDA (D.1), morphological state was verified by phase contrast microscopy (D.2) and fluorescence by epifluorescence microscopy (D.3).

τ_{act}, under 8.41 ± 1.21 min, t_{act}, with a activity recovery time scattering, S_{act}, of 2.44 ± 0.97 min. Afterwards, 99 ± 0% of active spores were able to outgrow, τ_{outgr}, in 96.54 ± 3.28 min, t_{outgr}, with a activity recovery time scattering, S_{outgr}, of 5.11 ± 0.64 min. The scattering of individual permeabilization time, S_p, was equal to 1.0 and was constant for all tested conditions.

After heat treatment at 85 °C during 12 min or at 95 °C during 2 min, 90% of the initial population was inactivated and was not able to form a colony on nutrient agar. The flow cytometry assays revealed that the time of permeabilization (t_p) was significantly extended to 35.33 ± 6.81 min or 38.46 ± 12.81 min, for 85 °C or 95 °C heat treatments respectively and a significantly lower proportion of permeable spore (τ_p) was observed, 54 ± 2%; or 70 ± 8% (ANOVA test $\alpha = 0.05$). Heat treatment also led to an extended time of activity recovery (t_{act}) which was estimated at 83.79 ± 36.6 min or 64.4 ± 25.7 min, and a lower proportion of active permeable spores (τ_{act}) was estimated at 52 ± 2% or 68.2 ± 1.2%, for 85 °C or 95 °C heat treatments respectively (ANOVA test $\alpha = 0.05$) (Table 1).

After heat treatment, *Bacillus weihenstephanensis* spores were incubated at pH 5.5. The number of permeable spores at pH 5.50 was

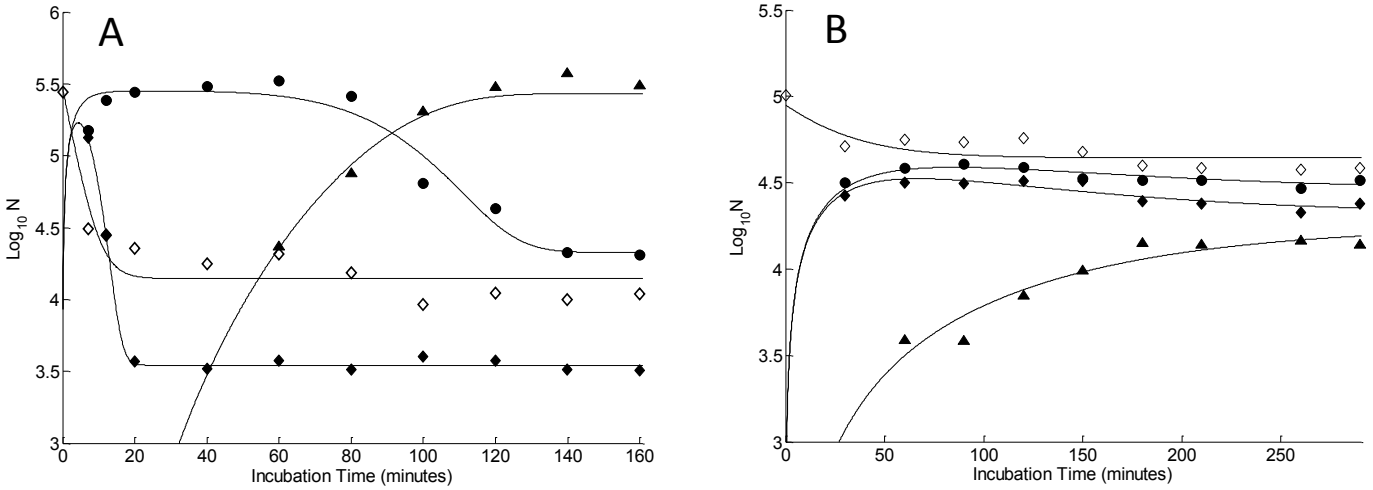

Fig. 2. Evolution of dormant spores (◊), permeable spores (♦), germinated spores (●) and vegetative cells (▲) regarding incubation time for (A) untreated spores incubated at pH 7.4 and (B) spores treated at 85 °C, 12 min, then incubated at pH 7.4. The lines represent the model presented in material and methods.

Table 1
Parameters describing permeabilization and activity recovery (mean value ± SD) for spores treated at 85 °C, 12 min, or 95 °C, 2 min and incubated at pH 7.4 or pH 5.5.

Heat treatment temperature	No heat treatment	Heat treatment 85 °C		Heat treatment 95 °C	
Incubation pH	7.4	7.4	5.5	7.4	5.5
τ_{perm} (%)	95 ± 1	54 ± 2	30 ± 11	70 ± 8	31 ± 2
t_{perm} (min)	3.32 ± 0.55	35.34 ± 6.81	40.90 ± 1.24	38.46 ± 12.8	80.26 ± 1.06
τ_{germ} (%)	99 ± 0	52 ± 2	11 ± 5	82 ± 4	12 ± 0
t_{germ} (min)	8.41 ± 1.20	83.79 ± 36.65	24.61 ± 1.9	64.36 ± 25.7	124.91 ± 42.02
S_{germ}	2.44 ± 0.97	1.71 ± 0.38[a]	5.8 ± 1.3	2.09 ± 1.38[a]	3.22 ± 1.90[a]

[a] Parameters not significantly different from the control condition, namely untreated spores incubated in pH 7.4.

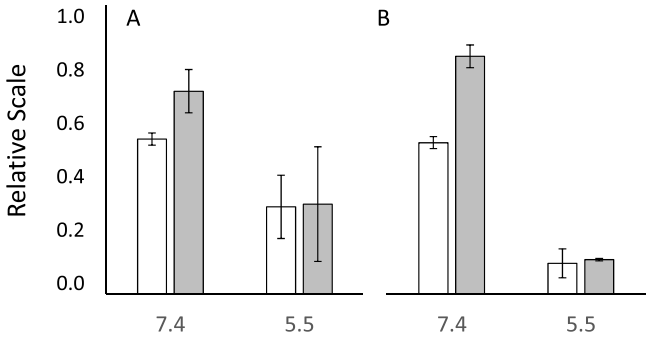

Fig. 3. Proportion of permeable spores (A) and germinated spores (B) for spores treated at 85 °C (white) or 95 °C (grey) and incubated at pH 7.4 or pH 5.5.

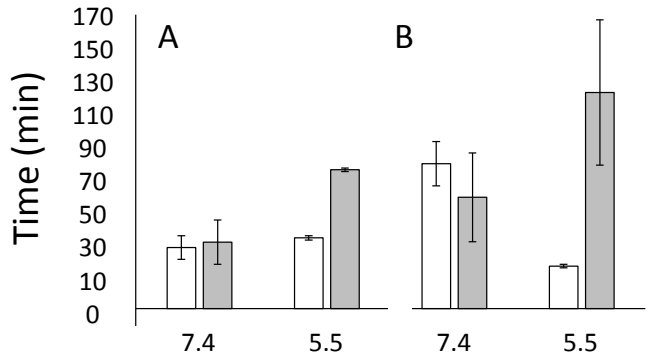

Fig. 4. Time of spore permeabilization (A) and spore germination (B) for spores treated at 85 °C (white) or 95 °C (grey) and incubated at pH 7.4 or pH 5.5.

threefold lower than in control conditions for both treatments and the time of permeabilization was extended 24.15 times or 12.8 times, for spores treated at 85 °C or 95 °C respectively. The number of active spores significantly decreased by an 8.33 fold or 9.25 fold, for spores treated at 85 °C or 95 °C respectively. Interestingly, the time of activity recovery was more impacted by an incubation at pH 5.5 after a heat treatment at 95 °C, as it was extended to 124.9 ± 42.0 min, than after a heat treatment at 85 °C as the germination was only extended to 24.6 ± 1.9 min. *B. weihenstephanensis* spores treated at 85 °C and incubated at pH 5.5 showed a time of activity recovery which was lower than the time of permeabilization and a scattering of activity recovery time significantly higher than in control conditions (ANOVA, $\alpha = 0.05$) (Figs. 3 and 4).

4. Discussion

The first step of germination consists in the release of CaDPA from the spore core which is partially rehydrated (Setlow, 2003). To achieve this first step, corresponding to the permeabilization step in the present work, no metabolism is necessary and the DNA can be reached by DNA intercalate markers (Setlow, 2007). Thus, spores which were in this state of partial rehydration were stained by LDS 751, binding the DNA, but the CFDA was not processed by esterase. The germination is completed by the hydrolysis of the cortex peptidoglycan by Cortex Lytic Enzymes (CLE) which are triggered by CaDPA or other unknown mechanisms in *Bacillus* spores (Setlow, 2007). Then spore core extends and metabolic activity can be observed as it was previously showed during *B. anthracis* spores germination using esterase activity as a marker of metabolic activity. Indeed, esterase activity could be observed in *B. anthracis* spores rapidly after the rehydration of the spore (Ferencko et al., 2004).

In previous works, germination process has been described by the discrimination between dormant spores (phase bright spores, or impermeable to markers) and permeable spores (phase dark spores, or permeable to markers) (Mathys et al., 2007; Pandey et al., 2013; van Melis et al., 2011). However, it has been showed that spores of *B. subtilis* can lose their refractivity, and that *G. stearothermophilus* spores can be permeable to some markers, such as Syto9, after a heat treatment and remain in this state for an extended period (Mtimet et al., 2017; Pandey et al., 2015). The actual state of the spores remaining permeable to Syto9 (or other molecules of Syto family) or remaining as phase dark spores after a heat treatment was unclear. The discrimination between inactive permeable spores and active permeable spores was necessary to determine the physiological state of the spores remaining phase dark after an extended period. This discrimination also helps to reach a better understanding of the heat treatment impact on spores, and a better quantification of this impact. The method developed in this work allowed the discrimination of inactive permeable spores and active permeable spores thanks to double staining with LDS 751 and CFDA. The spores appeared in red if they were only rehydrated, then considered as permeable. Then, the spores appeared red and green as they were permeable and showed an esterase activity. CFDA has been shown to efficiently stain bacterial activity (Hoefel et al., 2003). In this work, the proportion of CFDA positive cells corresponded to the number of cells able to form colonies on nutritive agar medium (data not shown).

The method developed in this work was applied to the monitoring of the physiological evolution of *Bacillus weihenstephanensis* KBAB4 spores after to different heat treatments. The impact of heat treatment could be quantified on spores treated at 85 °C during 12 min or at 95 °C during 2 min, then incubated at pH 7.4. These treatments inactivated 90% of the initial population, which was verified by classical cultural methods. Both heat treatments led to a significantly lower proportion of inactive permeable and active spores and an extended time of permeabilization and activity recovery. These observations were consistent with previous work in which spores of *Bacillus subtilis* presented a lower proportion of spores losing their refractivity or releasing their CaDPA (monitored by Raman spectroscopy) after heat treatment (Pandey et al., 2015; Wang et al., 2011). The time of outgrowth was also significantly extended and the proportion of vegetative cells was significantly lower after heat

treatment. Interestingly, two different heat treatment conditions led to different spore behaviors. Indeed, a heat treatment at 85 °C during 12 min led to a significantly lower proportion of permeable spores than a heat treatment at 95 °C during 2 min. This observation suggests different impact of heat treatment temperature and/or time on some proteins involving the induction of germination process as it was mentioned in previous works (Coleman et al., 2007; Setlow, 2013). In addition, the proportion of permeable spores able to recover a metabolic activity was lower after a heat treatment at 85 °C during 12 min. It is not clear if the temperature or the time of treatment, or the combination of both parameters, is the main factor of this difference.

After heat treatment, the spores were incubated in conditions allowing the growth (pH 5.5 and pH 7.40). The proportion of permeable spores was significantly lower when spores were incubated at pH 5.5 whatever the heat treatment. The binding of germinant on the germinant receptor might be faintly impacted by the environmental pH. The incubation pH had also a significant impact on proportion of active permeable spores - inner pH spore increases from ~6.5 to ~7.5 during germination-, mainly due to the presence of weak acids in rich media, as the one used in this work(van Beilen and Brul, 2013). Thus, a suboptimal environmental pH may have an impact on the recovery of metabolic activity within *Bacillus* spores. Interestingly, the time of permeabilization was significantly impacted by the incubation pH of 5.5 only after a heat treatment at 95 °C during 2 min. The underlying molecular mechanism(s) leading to this behavior (impact on binding step of germinant, CaDPA channel, etc.) remains unclear and further experiments must be necessary to unravel if this due to the temperature of treatment, the time of treatment, the pH of incubation or a combination of all or part of these parameters. Interestingly, for spores treated at 85 °C during 12 min then incubated at pH 5.5, 90% of the spores recovered an esterase activity after 25 min. However, a fewer part of refractive spores lately lost their permeability after 40 min but were not able to recover an activity in less than 250 min of incubation. The spores kept the ability to rehydrate but the environmental lower pH inhibited the metabolic activity or the heat treatment has inactivated key targets needed for the metabolism recovery. As previously specified, further experiments are necessary to unravel the relative implication of heat treatment and/or incubation conditions on the spore behavior in these conditions.

5. Conclusion

Flow cytometry associated with double staining, with LDS 751 and CFDA, allows a satisfactory discrimination of dormant spores, inactive permeable spores, active germinated spores and vegetative cells. The method developed in this work quantifies the number of spores in each stage over time, regarding the conditions the spores are subjected to. This method allowed distinguishing the impact of heat treatment and post-treatment incubation pH on the two first steps of germination: spore permeabilization and activity recovery. The heat treatment conditions, temperature and time of treatment, have an impact on spore permeabilization and germination as a treatment at 85 °C, 12 min, leads to a lower proportion of permeable spores and germinated spores compared to a heat treatment at 95 °C, 2 min. This can be due to the permeabilization process occurring during the heat treatment itself, or the fact that different treatment intensity affect different targets within the spore structure. A lower incubation pH after heat treatment lead to lower proportion of permeable and germinated spores in growth conditions. These results are of interest for food industry as it allows a better understanding of the impact process parameters (heat treatment intensity) or formulation (pH the spores can encounter after the heat stress) on spore ability to recover metabolic activity and to spoil the food product. Two different heat treatments have been investigated in this study and further work is needed to unravel whereas the temperature or the duration of heat treatment have the main impact on spore ability to recover a metabolic activity.

Acknowledgement

This research did not receive any specific grant from funding agencies in public, commercial, or not-for-profit sectors.

References

Abee, T., Groot, M.N., Tempelaars, M., Zwietering, M., Moezelaar, R., Voort, M., 2011. Germination and outgrowth of spores of *Bacillus cereus* group members: diversity and role of germinant receptors. Food Microbiol. 28, 199–208.

Baril, E., Coroller, L., Postollec, F., Leguerinel, I., Boulais, C., Carlin, F., Mafart, P., 2011. The wet-heat resistance of *Bacillus weihenstephanensis* KBAB4 spores produced in a two-step sporulation process depends on sporulation temperature but not on previous cell history. Int. J. Food Microbiol. 146, 57–62.

Coleman, W.H., Chen, D., Li, Y., Cowan, A.E., Setlow, P., 2007. How moist heat kills spores of *Bacillus subtilis*. J. Bacteriol. 189, 8458–8466.

Ferencko, L., Cote, M.A., Rotman, B., 2004. Esterase activity as a novel parameter of spore germination in *Bacillus anthracis*. Biochem. Biophys. Res. Commun. 319, 854–858.

Hoefel, D., Grooby, W.L., Monis, P.T., Andrews, S., Saint, C.P., 2003. A comparative study of carboxyfluorescein diacetate and carboxyfluorescein diacetate succinimidyl ester as indicators of bacterial activity. J. Microbiol. Meth. 52, 379–388.

Huet, S., Bouvier, A., Poursat, M.-A., Jolivet, E., 2010. Statistical Tools for Nonlinear Regression 2e: A Practical Guide with S-PLUS and R Examples, Springer Series in Statistics. Springer.

Mathys, A., Chapman, B., Bull, M., Heinz, V., Knorr, D., 2007. Flow cytometric assessment of *Bacillus* spore response to high pressure and heat. Innovat. Food Sci. Emerg. Technol. 8, 519–527.

Mtimet, N., Trunet, C., Mathot, A.-G., Venaille, L., Leguérinel, I., Coroller, L., Couvert, O., 2017. Walking dead: permeabilization of heat-treated *Geobacillus stearothermophilus* ATCC 12980 spores under growth-preventing conditions. Food Microbiol. 64, 126–134.

Mtimet, N., Trunet, C., Mathot, A.-G., Venaille, L., Leguérinel, I., Coroller, L., Couvert, O., 2015. Modeling the behavior of *Geobacillus stearothermophilus* ATCC 12980 throughout its life cycle as vegetative cells or spores using growth boundaries. Food Microbiol. 48, 153–162.

Pandey, R., Pieper, G.H., Beek, A.T., Vischer, N.O.E., Smelt, J.P.P.M., Manders, E.M.M., Brul, S., 2015. Quantifying the effect of sorbic acid, heat and combination of both on germination and outgrowth of *Bacillus subtilis* spores at single cell resolution. Food Microbiol. 52, 88–96.

Pandey, R., Ter Beek, A., Vischer, N.O.E., Smelt, J.P.P.M., Brul, S., Manders, E.M.M., 2013. Live cell imaging of germination and outgrowth of individual *Bacillus subtilis* spores; the effect of heat stress quantitatively analyzed with sporetracker. PLoS One 8, e58972.

Setlow, P., 2014. Germination of spores of *Bacillus* species: what we know and do not know. J. Bacteriol. 196, 1297–1305.

Setlow, P., 2013. Summer meeting 2013 – when the sleepers wake: the germination of spores of *Bacillus* species. J. Appl. Microbiol. 115, 1251–1268.

Setlow, P., 2007. I will survive: DNA protection in bacterial spores. Trends Microbiol. 15, 172–180.

Setlow, P., 2003. Spore germination. Curr. Opin. Microbiol. 6, 550–556.

Scherrer, B., Legendre, P., Beltangady, M., 2009. Biostatistique : Volume 2. Édition : 2e édition. ed. Gaetan Morin, Montréal.

Trunet, C., Carlin, F., Coroller, L., 2017. Investigating germination and outgrowth of bacterial spores at several scales. Trends Food Sci. Technol. 64, 60–68.

Trunet, C., Mtimet, N., Mathot, A.-G., Postollec, F., Leguerinel, I., Sohier, D., Couvert, O., Carlin, F., Coroller, L., 2015. Modeling the recovery of heat-treated *Bacillus licheniformis* Ad978 and *Bacillus weihenstephanensis* KBAB4 spores at suboptimal temperature and pH using growth limits. Appl. Environ. Microbiol. 81, 562–568.

van Beilen, J.W.A., Brul, S., 2013. Compartment-specific pH monitoring in *Bacillus subtilis* using fluorescent sensor proteins: a tool to analyze the antibacterial effect of weak organic acids. Front. Microbiol. 4.

van Melis, C.C.J., den Besten, H.M.W., Nierop Groot, M.N., Abee, T., 2014. Quantification of the impact of single and multiple mild stresses on outgrowth heterogeneity of *Bacillus cereus* spores. Int. J. Food Microbiol. 177, 57–62.

van Melis, C.C.J., Nierop Groot, M.N., Tempelaars, M.H., Moezelaar, R., Abee, T., 2011. Characterization of germination and outgrowth of sorbic acid-stressed *Bacillus cereus* ATCC 14579 spores: phenotype and transcriptome analysis. Food Microbiol. 28, 275–283.

Wang, G., Zhang, P., Setlow, P., Li, Y.-q, 2011. Kinetics of germination of wet-heat-treated individual spores of Bacillus species, monitored by Raman spectroscopy and differential interference contrast microscopy. Appl. Environ. Microbiol. 77, 3368–3379.