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**Cycling versus Continuous High Pressure treatments at moderate temperatures: effect on bacterial spores?**

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## Abstract

The advantage of using high pressure (HP) cycling treatment compared with continuous HP treatment was investigated for the inactivation of bacterial spores. The effects of parameters such as pulse number, pressure level, treatment temperature, compression and decompression rates, and time between pulses were evaluated. For this purpose, *Bacillus subtilis* and *B. cereus* spores ( $10^8$  and  $10^6$  CFU/mL respectively) were suspended in 2-(N-morpholino) ethanesulfonic acid (MES) buffer solution, tryptone salt (TS) buffer solution, or infant milk and treated by HP cycling at 300-400 MPa, at 38-60°C, for 1–5 pulses. Pressure cycling reduced the number of viable spores by 1.8 and 5.9 log respectively for *B. subtilis* and *B. cereus* species. Continuous HP treatments were performed at the same pressure and temperature for similar treatment durations. Our results showed that the spore inactivation ratio was correlated with the cumulative exposure time to pressure rather than to effects of the cycling process. Greater spore inactivation caused by HP cycling was observed only when faster compression and decompression rates were applied, probably due to adiabatic heating. A three-step kinetic model was developed, which seemed to support our hypothesis regarding the mechanisms of inactivation by pressure cycling and continuous HP treatments.

## Keywords

Bacterial spore; germination; inactivation; high pressure; cycling pressure; kinetic model.

## Industrial relevance

The resistance of bacterial spores to HP limits the industrial applications to refrigerated food products. In this study, we investigated the use of pressure cycling as a means to improve spore baroinactivation at moderate temperatures ( $T < 60^\circ\text{C}$ ). We showed that cycling pressure does not significantly increase bacterial spore inactivation in comparable treatment duration, but certainly increases material fatigue in HP vessels. Thus, under moderate temperature, cycling pressure treatment is not industrially relevant.

## Highlights

- The highest inactivation was 1.8 and 5.9 log for *B. subtilis* and *B. cereus* spores respectively.
- Spore inactivation was mainly related to the pressure holding time and not to the cycling effect.
- A three-step kinetic model with a good fit was developed for spore inactivation by pressure cycling.
- Slow compression kinetic during cycling pressure lead to higher inactivation due to holding time under pressure during compression time except for very rapid compression rate.

## 1. Introduction

Members of the genera *Bacillus* and *Clostridium* can form endospores, named hereafter as spores, primarily when nutrient sources are depleted, or are exposed to environmental stresses such as accumulation of organic acids, exposure to oxygen and chemicals agents that affect bacterial growth (Al-Hinai, Jones, & Papoutsakis, 2015; Gauvry et al., 2017). Bacterial spores are metabolically dormant forms that display great resistance to various stresses such as heat, chemical agents, and physical processes (Setlow, 2006).

Because of these extreme resistance properties, bacterial spores can be involved in food spoilage and foodborne illness (André, Vallaey & Planchon, 2017). Spoilage due to sporeforming bacteria causes high economic losses in food and feed industry. Food spoilage is characterized by variation of the physicochemical properties (texture, odor, pH and / or gas production) depending on food matrices when spores return to vegetative state. For shelf-stable products, high temperatures and/or long holding time thermal treatments are necessary to inactivate spores in food sterilization processes (Wells-Bennik et al., 2016). However, such treatments can decrease the nutritional value and sensory properties of food. Otherwise, the use of hurdle technologies can increase the shelf-life of refrigerated food products.

Among alternative soft technologies, the high-pressure (HP) processing is an established food processing technique for inactivating vegetative forms of pathogenic and spoilage bacteria and maintaining food quality (Sevenich & Mathys, 2018). However, the destruction of spores by HP processing remains a challenge because of the high resistance of spores to pressure. HP is known to initiate spore germination, causing their inactivation under pressure, but fails to induce germination and inactivation of the entire spore population in order to provide food sterilization. The effect of HP on spore germination is dependent on the pressure level, holding time, and temperature range (Reineke, Mathys, Heinz, & Knorr, 2013). At lower pressures (< 300 MPa) and temperatures around 30-50°C, physiological germination by the

activation of nutrient germinant receptors (GR) arises in *Bacillus* spores, which is similar to nutrient-triggered germination (Black et al., 2005; Delbrück, Zhang, Heydenreich, & Mathys, 2021a; Lenz & Vogel, 2015; Reineke et al., 2013; Zhang, Delbrück, Off, Benke, & Mathys, 2020). At higher pressures (> 400 MPa), *Bacillus* spore germination is induced by the opening of channels that allow the loss of dipicolinic acid (DPA) (Black et al., 2007; Lenz & Vogel, 2015; Margosch, Gänzle, Ehrmann, & Vogel, 2004; Paidhungat et al., 2002; Reineke et al., 2013). Mechanisms of HP induction of *Clostridia* spore germination are almost similar to *Bacillus* species, except for *Clostridium* species lacking GR such as *Clostridium difficile* (Doona et al., 2016a; Setlow, Wang, & Li, 2017).

HP treatment (> 600 MPa) combined with higher temperatures (> 60°C) increase the ratio of spore inactivation (Reineke, Mathys, & Knorr, 2011). In this case, spore inactivation mechanisms remain hypothetical, but inactivation seems clearly related to DPA release, core hydration, and temperature inactivation of heat-sensitive germinated spores. Even at such temperatures, DPA release is probably related to germination induction. Essential oils, such as carvacrol, which are known to inhibit spore germination, are able to protect spores from high-pressure high-temperature inactivation (Corthouts & Michiels, 2016; Luu-Thi et al., 2015).

HP cycling treatment consists of applying several successive pressure pulses with decompression phases between them. The effects of pressure cycling parameters (compression and decompression rates, number of pulses, pressure holding time, and temperature) has been extensively studied for vegetative bacteria and for yeast inactivation in foods (Chapleau et al., 2006; Donsì, Ferrari, & Maresca, 2007; Noma, Shimoda, & Hayakawa, 2002; Rivalain et al., 2012). However, contradictory conclusions have been reported, especially regarding the influence of compression and decompression rates.

In the same way, several groups have been interested in the application of pressure cycling as a means to improve spore inactivation (Demazeau et al., 2018; Furukawa, Nakahara, &

Hayakawa, 2000; Hayakawa, Kanno, Yoshiyama, & Fujio, 1994; Obaidat, Yu, Aljawhiri, & Macgregor, 2015; Ratphitagsanti, Ahn, Balasubramaniam, & Yousef, 2009). Pressure cycling was more effective than continuous pressure in terms of spore inactivation of *Bacillus subtilis* (200, 300 and 400 MPa, 25, 35, 45 and 55°C), *Geobacillus stearothermophilus* (400 and 600 MPa, 70°C) and *B. amyloliquefaciens* (600 MPa, 105°C) (Furukawa, Shimoda, & Hayakawa, 2003; Hayakawa et al., 1994; Ratphitagsanti et al., 2009). Rapid decompression during pressure cycling (200 MPa, 75°C) can increase *B. stearothermophilus* spore inactivation by permeabilization (Hayakawa et al., 1998). On the other hand, Ratphitagsanti et al. (2009) studied the effects of compression rate on the inactivation of *B. amylolequefaciens* spores and observed greater inactivation with slow compression (3.75 MPa/s; 5.7 log inactivation) than for rapid compression (18.06 MPa/s; 1 log inactivation) at a pressure level of 600 MPa at 105°C. Recently, Demazeau et al. (2018) established an HP cycling process (350 MPa, 38°C) that can inactivate 6 log (CFU/mL) of *B. cereus* spores inoculated into breast milk.

The objective of this study was to clarify the specific effects of HP cycling treatment and to identify treatment parameters that optimize bacterial spore inactivation in *B. cereus* and *B. subtilis* species, a pathogenic food spoilage spore and a model spore respectively. The HP inactivation curve is the result of several dynamical processes that give rise to a nonlinear response. The application of nonlinear models, such as the quasi-chemical kinetic (QCK) model, based on a two-step mechanism: conversion of dormant spores to germinated spores and then to inactivated spores, has been described in recent reviews (Doona et al., 2016b; Doona et al., 2017). We used an equivalent approach to model the kinetics of cycling pressure inactivation of *B. subtilis* spores using 1-5 pulses at 350 MPa and 40°C. Our purpose was to identify the causes and mechanisms of inactivation in order to assess the industrial interest of HP cycling treatments for the inactivation of bacterial spores.

## 2. Materials and Methods

### 2.1. Strains and spore productions

*Bacillus subtilis* PS533 (Department of Molecular, Microbial, and Structural Biology, University of Connecticut Health Center, USA) is a wild-type strain, a derivative of the isogenic strain PS832. PS533 carries the plasmid pUB110, which encodes resistance to kanamycin. *Bacillus subtilis* cells were resuscitated from frozen glycerol stock on LB medium with kanamycin (Luria Broth, 15 g.L<sup>-1</sup>, Sigma-Aldrich, Saint-Quentin Fallavier, France; bacteriological agar type E, 15 g.L<sup>-1</sup>, Biokar, Beauvais, France). Spores were prepared at 37°C in 2 × Schaeffer's-glucose liquid medium. A preculture was used to inoculate the medium at a ratio of 1:10 in a baffled flask before incubation at 37°C. Spore suspensions were purified as described by Setlow (2018) and appeared to contain > 95% dormant spores by phase contrast microscopy. Spores were then stored at 4°C in sterile distilled water for a maximum of one month until use.

For this study, a strain of *B. cereus* KBAB4 from the collection of INRAE's was also used. Suspensions of *B. cereus* spores were prepared according to the French Standard Method NF T 72-231 as summarized below. *Bacillus cereus* suspensions were incubated at 37°C in brain heart infusion (BHI; Biokar Diagnostics). The sporulation steps consisted of the use of sporulation medium (10 g.L<sup>-1</sup> beef extract, 2 g.L<sup>-1</sup> yeast extract, 0.04 g.L<sup>-1</sup> MnSO<sub>4</sub>·H<sub>2</sub>O, and 15 g.L<sup>-1</sup> agar) and incubation for 2-5 days at 37°C. Sporulation was monitored by phase contrast microscopy, and spores were harvested when > 95% of the bright phase spores were observed. Spores were harvested by flooding the agar surface with sterile distilled water before being transferred to a sterile centrifugation tube. Spore suspensions were centrifuged (4,000 × g, 20 min, 4°C), the pellet was washed three times and finally resuspended in 20 mL of sterile distilled water. A heat treatment (80°C for 10 min) was performed and spores were suspended in 0.2-µm filtered 70% ethanol (filter: cellulose acetate, Sartorius, Dourdan,



France; ethanol: Elvetec, Nemours, France) for 1 h in order to inactivate vegetative cells. Spores were then washed three times by successive centrifugation ( $3,600 \times g$ , 15 min,  $4^{\circ}\text{C}$ ) and suspended in sterile distilled water at  $4^{\circ}\text{C}$ . Spores were counted by pouring 1 mL of each suspension onto BHI agar plates (Biokar Diagnostics) according to the French standard NF V 08–602. *Bacillus cereus* was incubated at  $37^{\circ}\text{C}$  for 48 h. The concentration of the final spore suspensions was standardized at  $10^7$  CFU/mL, and suspensions were stored at  $4^{\circ}\text{C}$  for a maximum of one month.

## 2.2. Spore preparations for HP treatment

For each experiment, *B. subtilis* spores were suspended in a pressure-stable 2-(N-morpholino) ethanesulfonic acid (MES, Sigma Aldrich) buffer solution to obtain a final spore concentration of approximately  $10^8$  spores/mL. MES buffer solution was chosen for HP experiments because its pH varies slightly with pressure and temperature ( $\Delta\text{pKa}/^{\circ}\text{C} = -0.011$ ; pH varies from 5.5 to 6.5 between 10 and 1000 MPa at  $25^{\circ}\text{C}$ ) (Bruins, Matser, Janssen, & Boom, 2007). One mL of the spore suspension was transferred to a sterile polyethylene pipet (Dominique Dutscher, Issy-les-Moulineaux, France). For experiments on *B. cereus*, samples were prepared by a 90% dilution of the initial spore suspensions in tryptone salt (TS) buffer solution (Biokar Diagnostics). Then, each spore suspension (0.5 mL) in TS was heat-sealed into polyethylene pouches (polyethylene transfer pipet, Dominique Dutscher).

*Bacillus subtilis* spores were then treated with HP in a Top Industrie HP vessel of  $20 \text{ cm}^3$  (Top Industrie, Vaux le Penil, France). Experiments on *B. cereus* spores were performed using a different HP machine. This instrument was a 600-MPa HP vessel of  $30 \text{ cm}^3$  (Top Industrie). Both HP systems were equipped with a double-jacket, and water served as the pressure-transmitting fluid. The internal temperature of the pressure chamber was maintained using a water bath (Minisat 240, Huber, Germany) connected to the double wall of the pressure chamber and monitored using a Pt100 thermocouple (Top Industrie). The water bath

temperature was set to limit adiabatic heating. The pressure and temperature during HP treatment were also recorded using InstruNet World (iW) software (GW Instruments, Charlestown, USA).

For each strain, zero-time samples were taken to determine initial spore concentrations before treatment. Pouches were placed in the HP vessel 5 min before treatment to allow the temperature to equilibrate. The samples were then treated at the desired pressure-temperature couple and immediately immersed in iced water.

### 2.3. Enumeration of HP-treated spores

After HP treatment, spores were serially diluted 1/10 in PBS and aliquots were plated on LB medium for *B. subtilis* spores or on BHI medium for *B. cereus* spores. To determine heat-sensitive *B. subtilis* spores, HP-treated spores were heated at 80°C for 10 min to inactivate HP-germinated spores. After heat treatment, pressure and heat-treated (HPHT) spores were serially diluted in PBS and spotted onto LB medium. Plates were incubated at 37°C until colonies appeared, which were then counted.

Inactivation and germination were expressed as follow:

$$\text{Inactivation} = \log_{10} \left( \frac{N_0}{N_{HP}} \right)$$

$$\text{Germination} = \log_{10} \left( \frac{N_{HP}}{N_{HPHT}} \right)$$

$N_0$ : initial live spore concentration as given by colony-forming unit (CFU/mL),  $N_{HP}$ : HP-treated spores (live spore concentration after HP treatment), and  $N_{HPHT}$ : HPHT-treated spores (live spore concentration after HP and HT treatments).

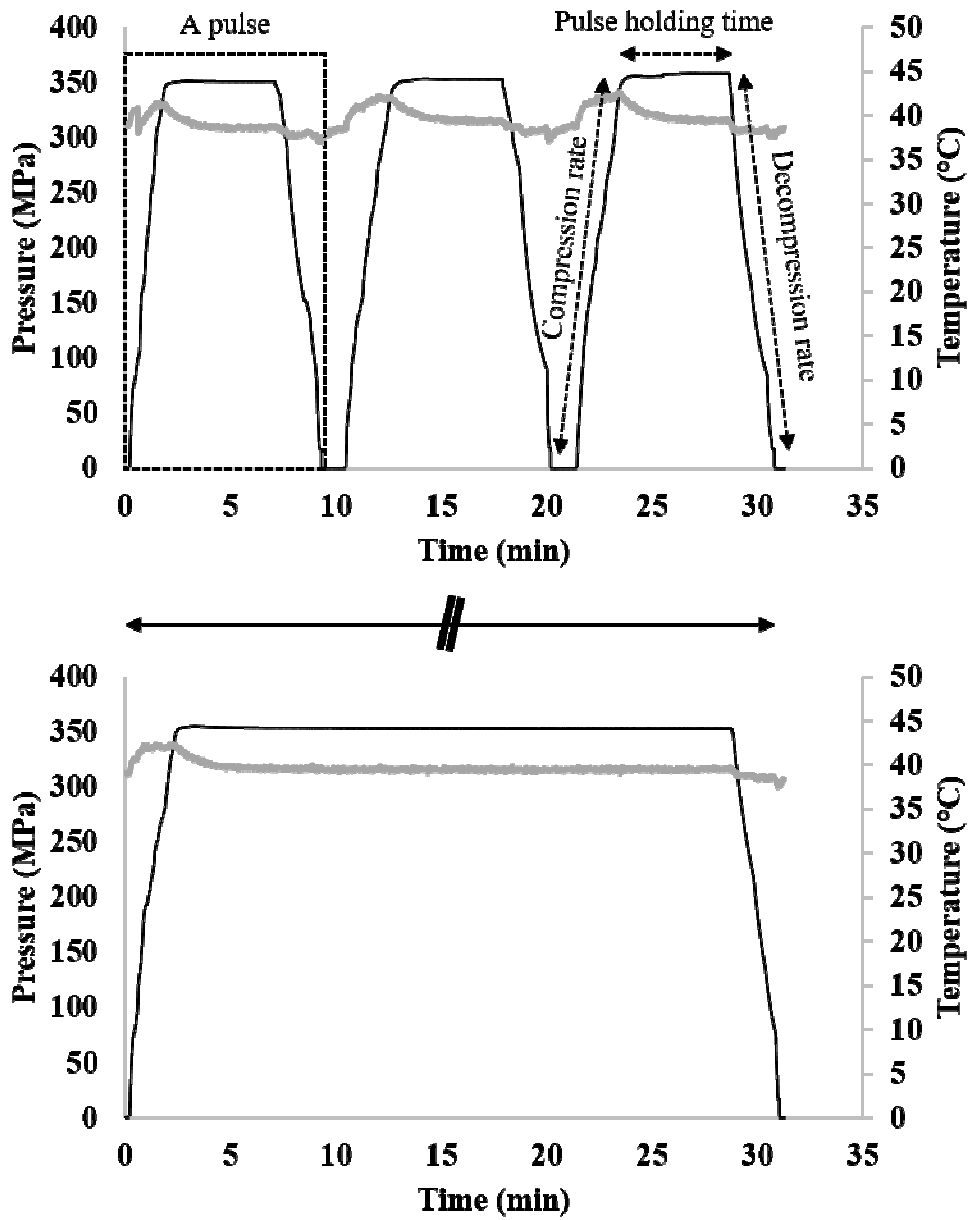
### 2.4. HP treatment characteristics

HP cycling treatments were characterized by the pressure level (350 MPa for *B. subtilis*; 300, 350, or 400 MPa for *B. cereus*), the pulse number (between 1 and 5 pulses) with a pulse

holding time of 5 min, and the time between pulses (0, 1, 5 min). These cycling treatments were also characterized by the compression rate (CR) to reach the target pressure and the decompression rate (DR) to return to atmospheric pressure (*B. subtilis*: CR = 2.5 MPa.s<sup>-1</sup>, DR = 2.5 MPa.s<sup>-1</sup> unless otherwise stated; *B. cereus*: CR = 2.5 MPa.s<sup>-1</sup>, DR < 3 s). Temperature of the treatment during the plateau (40°C for *B. subtilis*; 38, 50, and 60°C for *B. cereus*) was strictly controlled so that the temperature variation was ± 3°C. For the treatment of *B. subtilis* spores with rapid compression and decompression rates, the temperature variation was greater but did not exceed 8°C.

Continuous HP treatments were characterized by (1) a compression, (2) a pressure holding time and (3) a decompression, for a total duration corresponding to pressure cycling treatments including times between pulses, compression, and decompression times.

Characteristics of both treatments are presented in **Fig. 1**.

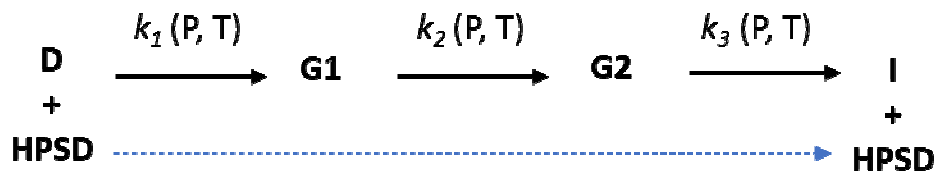


**Figure 1:** Characteristics of cycling and continuous HP treatments. Pressure recording is plotted as a black line and temperature as a gray one.

## 2.5. Statistical analysis and modeling approaches of spores inactivation

All experiments were performed in triplicate. A bilateral analysis of variance (ANOVA) comparing continuous and cycling HP treatments was systematically performed using R v.3.5.1 software (R Core Team (2018)). P-values below 0.05 were classified and termed as significantly different in the text and highlighted with a star index.

A quasi-chemical kinetic model was used to represent the non-linear dynamics of *B. subtilis* spore inactivation by HP treatment at 350 MPa, 40°C, with 1-5 pulses. Similar to the approach developed by Doona et al. (2016), this model is based on a simplified mechanism with successive stages describing the germination sequence responsible for spore inactivation by the HP process (Doona, Feeherry, Ross, & Kustin, 2016). Each transition follows first-order kinetics characterized by a parameter  $k_i$  [ $i = 1-3$ ] ( $s^{-1}$ ). In the proposed mechanism (**Fig. 2**), HP treatment induces the conversion of dormant spores (**D**) to germinated spores at stage 1 (**G1**), followed by germinated spores at stage 2 (**G2**), which can be inactivated by pressure treatment (**I**). Spores that never germinate on the timescale of the experiment are considered to be HP superdormant spores (**HPSD**), as described by Delbrück and colleagues (Delbrück, Zhang, Hug, Trunet, & Mathys, 2021b).



**Figure 2:** Inactivation model based on the spore germination sequence wherein  $D + HPSD$  represents the initial population composed of dormant ( $D$ ) and superdormant spores ( $HPSD$ ),  $G1$  represents germinated spores at stage 1,  $G2$  germinated spores at stage 2, and  $I$  inactivated spores by the treatment.  $k_1$ ,  $k_2$  and  $k_3$  are model parameters ( $s^{-1}$ ).

The set of ordinary differential equations (ODEs) used was (Eq1):

$$(Eq1) \left\{ \begin{array}{l} \frac{dD}{dt} = -k_1 D \\ \frac{dG1}{dt} = k_1 D - k_2 G1 \\ \frac{dG2}{dt} = k_2 G1 - k_3 G2 \\ \frac{dI}{dt} = k_3 G2 \end{array} \right.$$

The  $k_1$ ,  $k_2$ , and  $k_3$  rates refer to the three steps described in **Fig. 2** and behave differently depending on the pressure (P) and temperature (T) during HP treatment. This pressure dependence was obtained by optimization and based on references in the literature (Heinz & Knorr, 1996; Pandey et al., 2013). Because  $k_1$ ,  $k_2$ , and  $k_3$  depend on pressure, the model is non-linear when these parameters vary. Therefore, we obtained a nonlinear ODE system. Model optimization was carried out using MATLAB® software R2018a (The Mathworks, Inc., Natick, Massachusetts, USA) from the experimental data ( $N_{HP}$  and  $N_{HPT}$ ) obtained with HP continuous treatments. MATLAB was used for numerical integration of the previous ODE system and model optimization. Unconstrained multivariate optimization was used to minimize the sum of squared deviations (SSD) between the model and experimental data. The accuracy of the model fit was assessed using the minimization of this sum of squared deviations.

From the experimental data ( $N_0$ ,  $N_{HP}$ , and  $N_{HPT}$ , refer to Section 2.3), the initial conditions and the values of D, G1, G2, and I were deduced for different experimental times as follows (Eq2):

$$(Eq2) \left\{ \begin{array}{l} D = N_{HP} - HPSD \\ G1 + G2 = N_{HP} - N_{HPT} \\ I = N_0 - N_{HF} \end{array} \right.$$

The model parameters to be optimized were  $k_1(P,T)$  and  $k_2(P,T)$  kinetics, and HPSD.

### 3. Results and Discussion

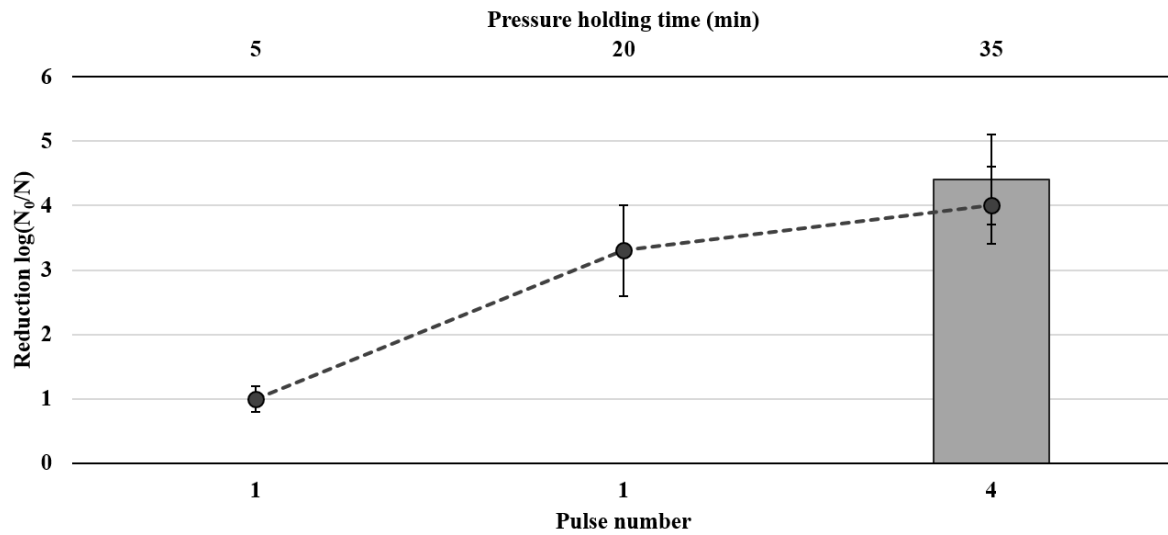
#### 3.1. Application of pressure cycling on *B. cereus* spores in infant milk

A first set of experiments was intended to reproduce the successful treatment proposed by Demazeau et al. (Demazeau et al., 2018) and to compare this with continuous HP treatment. The conditions were maintained as close as possible to those of the original protocol.

*Bacillus cereus* spores were suspended in infant milk ( $10^6$  spores/mL) (Gallia Calisma®, Limonest, France) and treated at 350 MPa, 38°C, 4 pulses of 5 min, with 5 min between pulses. Three continuous HP treatments were assessed as controls: a single HP treatment of 5 min ; a single HP treatment of 20 min corresponding to the total duration under pressure of the cyclic treatment ( $d = 4 \times 5$ ) ; and a single HP treatment of 35 min corresponding to the total duration of the cycling treatment including times between pulses ( $d = 4 \times 5 + 3 \times 5$ ).

The results displayed in **Fig. 3** show that increasing the holding time at 350 MPa on continuous treatment led to higher inactivation from 1 to 4 log for treatments of 5 and 35 min, respectively. At the same pressure, it appeared that the use of a cycling treatment increased spore inactivation to 4.4 log versus 4.0 log for a continuous treatment of 35 min with no significant difference ( $p > 0.05$ ). As the increase was not significant, it appears that the efficiency is due to the total duration of the treatment and not to the use of pressure cycling treatment. Under similar conditions, Demazeau and co-workers (Demazeau et al., 2018) obtained total inactivation of *B. cereus* spores (initial population of  $1.5 \times 10^6$  spores/mL) inoculated in breast milk, which represents a minimum inactivation ratio of 6.18 log. These differences may be explained by the different treatment methods, strains, and cultivation conditions. However, Demazeau et al. did not perform continuous HP treatment as a control. In our study, the cycling pattern employed did not significantly increase HP efficiency, which is important for *B. cereus*. Further experiments were performed on *B. subtilis* spores with

varying cycling parameters to clarify whether the cycling pattern could significantly increase the efficiency of HP treatment on bacterial spore inactivation.



**Figure 3:** Inactivation of *B. cereus* spores in a suspension of infant milk after pressure treatments. Bar: 4 pulses × 5 min, 350 MPa, 38°C, with a time between pulses of 5 min. Dashed curve: continuous HP treatments for 5, 20, and 35 min. Error bars represent SD calculated from independent triplicates.



### 3.2. Effect of pulses number on spore inactivation-germination

Germination and inactivation of *B. subtilis* spores were evaluated after pressure cycling with an increase in pulse number (from 1 to 5) at 350 MPa for 5 min at 40°C, with a time between pulses of 5 min. The inactivation of *B. cereus* spores was also evaluated for the same increase in pulse number at 300 MPa, with a pulse holding time of 5 min and a time between pulses of 1 min at 50 and 60°C. Germination was not measured with *B. cereus*. The results are presented in **Fig. 4** for *B. subtilis* and those for *B. cereus* in **Fig. 5**. Cycling treatment results were plotted in columns as a function of pulse number, while control continuous HP treatments were plotted with dots and lines as a function of treatment time. The time of each control continuous HP treatment was chosen to correspond exactly to the complete time of the cycling treatment (pulses and time between pulses). Thus, for one pulse, the cycling and continuous HP treatments were the same. Spore inactivation is plotted in dark gray columns (or black dashed line for the continuous HP treatment). For *B. subtilis*, spore germination after the same pressure treatment evaluated by heat treatment (80°C, 10 min) is also represented (see Material and Methods, section 2.3).

First, regarding the effect of pulse number, inactivation of *B. subtilis* spores was higher when the number of pulses was increased (**Fig. 4**). This inactivation reached 1.82 log for a treatment at 350 MPa at 40°C, with 5 pulses of 5 min. At the same time, spore germination ratio decreased with increasing pulse number from 1.33 log for 1 pulse to 0.08 log for 5 pulses. The first pressure pulse was sufficient to induce a significant proportion of spores to germinate (1.5 log). Then, the subsequent pressure pulses inactivated the germinated spores. **Fig. 4** compares pressure cycling treatments versus continuous HP treatments with holding times corresponding to the total duration of each pressure cycling treatment. Similarly, with cycling treatment, inactivation of *B. subtilis* spores increased with holding time ( $p < 0.05$ ) (1.76 log after 68 min of treatment), whereas the ratio of germinated spores decreased at the same time.

Pressure cycling treatment appeared to be more efficient for *B. subtilis* spore inactivation with only 2 or 3 pulses ( $p < 0.05$ ). For five pulses, no significant difference was observed with continuous HP treatments ( $p > 0.05$ ). Under these conditions (350 MPa, 40°C), inactivation seems limited to approximately 2 log, regardless of the time and the process (cycling or not). This was confirmed in an experiment carried out over a prolonged period (115 min 350 MPa 40°C, *B. subtilis* spore inactivation 1,9 log, data not shown).

As shown in **Fig. 5**, *B. cereus* spores were less resistant than *B. subtilis* spores, and inactivation from 2.7 to 5.8 log was obtained for treatment at 300 MPa and 50 or 60°C for 5 to 29 min. However, inactivation of *B. cereus* at 50°C exhibited the same trend. There was an effect of treatment time and pulse number on spore inactivation with or without cycling pattern ( $p < 0.05$ ). There was no cycling effect regardless of the number of pulses ( $p > 0.05$ ). At 60°C, spore inactivation was accelerated by 4.6 log after the first pulse of 5 min, while only 2.7 log were achieved at 50°C. Increasing the number of pulses and/or treatment time did not increase the extent of inactivation ( $p > 0.05$ ). There was no cycling effect at 60°C ( $p > 0.05$ ), as observed earlier at 50°C. Interestingly, increasing the temperature from 50 to 60°C greatly accelerated the treatment effect but did not increase the maximum inactivation (5.84 at 50°C vs. 5.4 at 60°C) on *B. cereus* spores.

Thus, the results presented in **Fig. 4 and 5** based on these two species (*B. subtilis* and *B. cereus*) demonstrated that increasing the number of pulses caused an increase in spore inactivation, whereas continuous HP treatments resulted in similar inactivation (for pulse numbers  $> 3$  for *B. subtilis* spores). The cycling effect, if it exists, would compensate for the time between pulses. However, other studies have already observed a cycling effect; Furukawa et al. increased pulses with the same treatment time (30 min) at 25, 35, and 45°C and observed cycling and a pulse number effects on *B. subtilis* spores, especially at 35°C (Furukawa et al., 2003). The inactivation ratios obtained were higher than those of the present

results (up to 6 log at 400 MPa, 45°C, 30 min), probably due to a more sensitive bacterial strain. It is interesting to note that 25 min of treatment was the only treatment where a cycling effect (two pulses) was observed, as in the present results. An increase in spore inactivation with pulse number has also been observed for *Geobacillus stearothermophilus* (Hayakawa et al., 1994) and *Alicyclobacillus acidoterrestris* (Sokołowska, Skąpska, et al., 2012). Demazeau et al. (2018) claimed to inactivate all spore populations (> 4 log) of *B. cereus* in breast milk with four pulses of 5 min at 350 MPa and 38°C. No comparison with continuous HP treatment was performed. Conversely, Obaidat et al. (2015) compared the inactivation obtained by pressure cycling (50 pulses of 90 s, 80 MPa, 38°C) to those obtained by continuous pressure (85 min at 80 MPa, 38°C) for the same exposure time to pressure and obtained the same inactivation of *B. subtilis* spores (1.91 log for cyclic treatment and 2.22 for continuous treatment).

Thus, our data suggest that, during an HP cycling treatment, the increase in spore inactivation with the number of pulses could be due to the cumulative exposure time to pressure rather than to the effect of the cycling process itself.

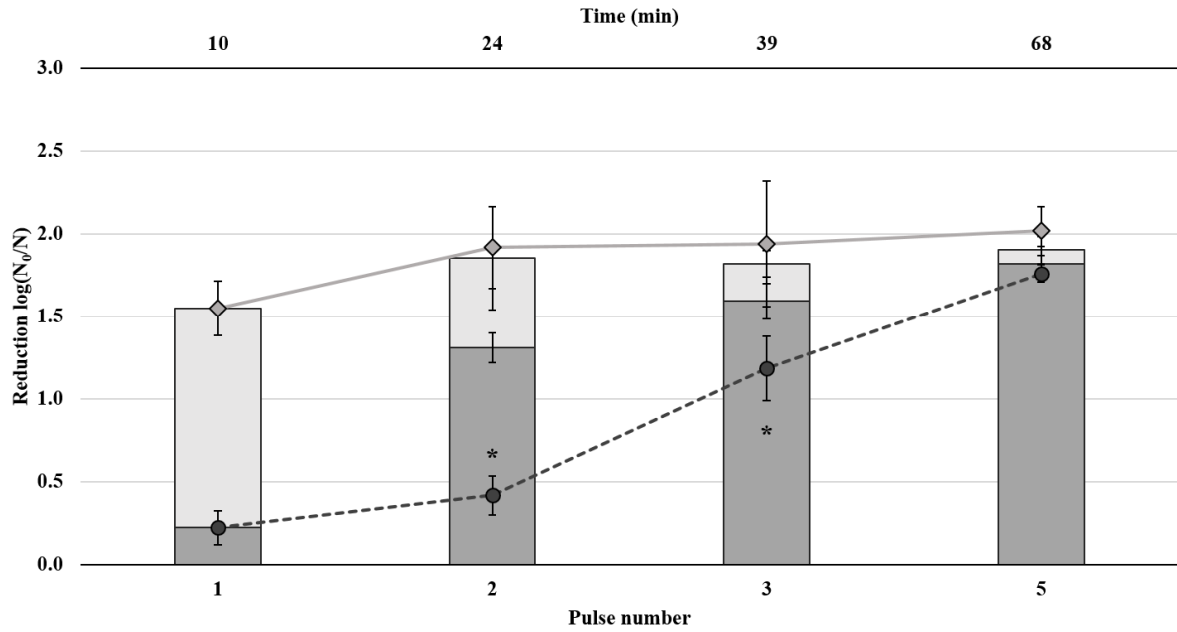
It is established that moderate HP treatment (< 350 MPa at temperatures < 50°C) can activate germination receptors (GRs) and induce physiological germination of *Bacillus* spores (Black et al., 2005). These two stages characterize the germination process of *Bacillus* spores. Initially, GR activation leads to the release of Ca-DPA via SpoVA channels, resulting in partial protoplasm rehydration (stage 1) and loss of heat resistance. Ca-DPA excretion triggers progression into stage 2 of germination, which consists of hydrolysis of the cortex by cortex lytic enzymes (CLEs). At the end of stage 2, the protoplasm is entirely hydrated (Setlow, 2014) and the spores lose their resistance to radiation, chemicals, and pressure.

In this study, 95% (1.33 log) of *B. subtilis* spores germinated after one HP pulse (**Fig. 4**). Subsequent pulses or more time under pressure for continuous treatments had a greater effect

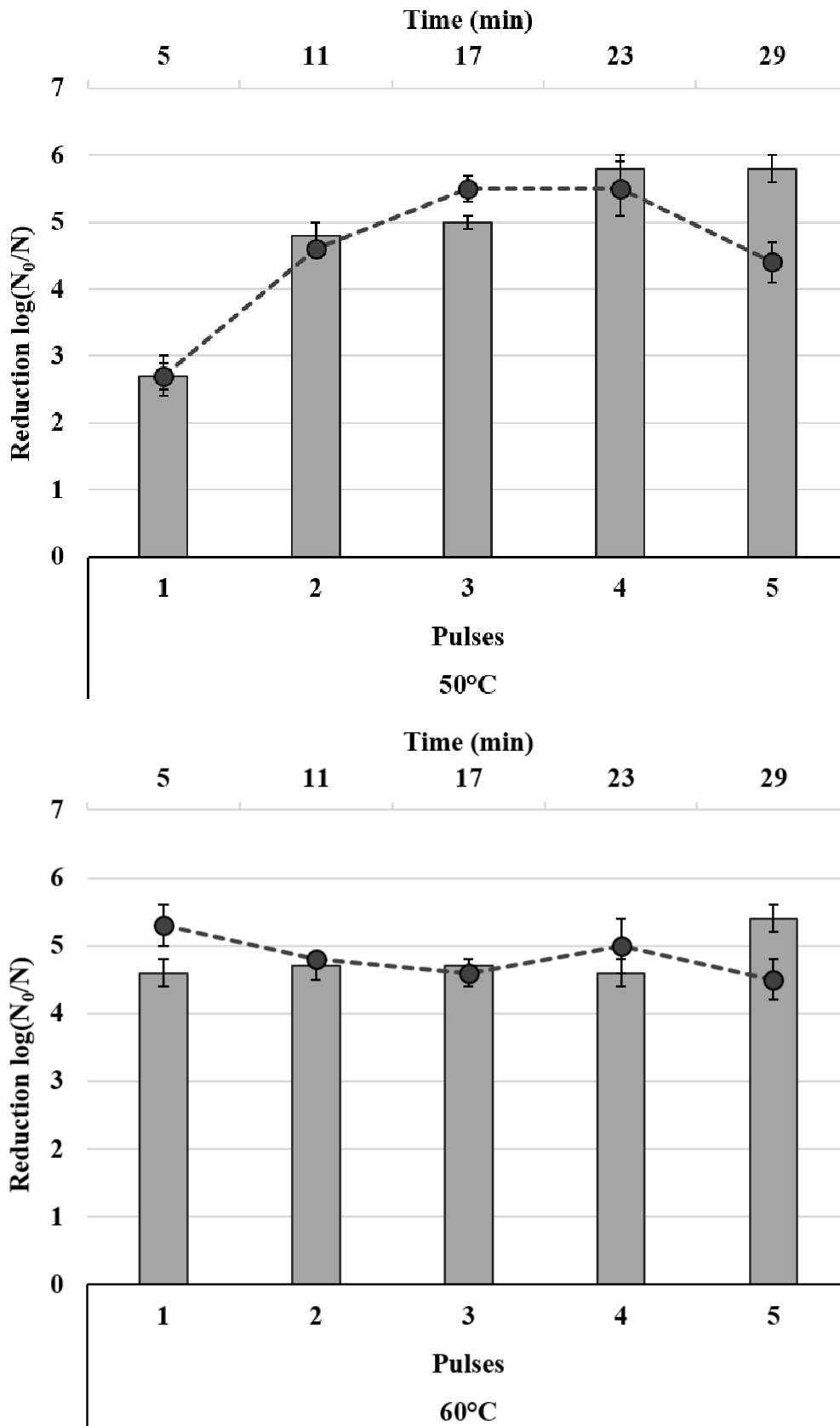
on inactivation of germinated spores than on germination itself. Cycling treatment does not seem to accelerate germination nor affect the germination ratio or the superdormant population.

*Bacillus cereus* was more efficiently sensitized by pressure; approximately 5 log of spores were inactivated at 50°C after two pulses for a cycling treatment or after equivalent continuous HP treatment. At 60°C, spores were inactivated after the first pulses probably because 60°C is lethal for germinated spores at stage 1, whereas at 50°C, germinated spores need to reach stage 2 to be affected by pressure treatment. The maximum spore inactivation, which represents the ability of pressure to induce germination, lay between 5 and 6 log and depended neither on temperature nor cycling pattern.

*Bacillus subtilis* is a mesophilic organism producing spores with a higher resistance to temperature than *B. cereus* which is a psychrotrophic species (Amador Espejo, Hernández-Herrero, Juan, & Trujillo, 2014). This resistance difference could be attributed to GR activity and/or triggering capacity although the germination mechanisms are the same for both species (Garcia, der Voort, & Abee, 2010). This could also explain the difference in pressure resistance among species of *Bacillus* (Kong, Doona, Setlow, & Li, 2014; Margosch, Gänzle, Ehrmann, & Vogel, 2004). Moreover, Rose et al demonstrated a link between the sporulation conditions and the germination and resistance properties: spores produced in liquid media germinate more rapidly and show greater sensitivity to heat and chemical treatments than spores produced on plates (Rose et al., 2007). In our study, *B. cereus* spores were produced on solid media but remain more sensitive to pressure than *B. subtilis* spores produced in liquid media.



**Figure 4:** Effect of pulse number (1, 2, 3 or 5) on germination and inactivation of pressure treated *B. subtilis* spores. *Bacillus subtilis* spores were suspended in MES buffer solution and treated at 350 MPa, 40°C, pulse holding time of 5 min, and time between pulses of 5 min. Curves: inactivation (black dashed curve) and germination (gray solid curve) of spores treated by continuous HP treatments. Bars: inactivation (dark gray bars) and germination (light gray bars) of spores treated by pressure cycling. Error bars represent SD calculated from independent triplicates; (\*) indicate statistically significant difference ( $p < 0.05$ ) between the equivalent continuous and cycling HP treatments.



**Figure 5:** Effect of pulse number (1, 2, 3, 4 or 5) on inactivation of pressure treated *B. cereus* spores. The black dashed curve represents inactivation of spores treated by continuous HP treatments. Gray columns represent inactivation of spores treated by pressure cycling. *Bacillus cereus* spores were suspended in TS buffer solution and treated at 300 MPa, 50 or

*60°C, pulse holding time of 5 min, time between pulses of 1 min. Error bars represent SD calculated from independent triplicates.*

### 3.3. Effect of time between pulses on spore inactivation-germination

Another parameter specific to cycling treatment is the time at atmospheric pressure between pulses. The effect of this parameter has rarely been tested in previous studies and is relatively short (i.e., 5 min (Sokołowska, Skąpska, et al., 2012)) or not documented (Furukawa et al., 2003; Hayakawa et al., 1994) and could play a role in spore germination.

*Bacillus subtilis* PS533 spores were treated by pressure cycling at 350 MPa, at 40°C, with 3 pulses of 5 min each, or 5 pulses of 5 min each, with different times between pulses (0, 1, and 5 min). The results are shown in **Fig. 6**. As observed earlier, spore inactivation increased with increasing pulses number regardless of the time between pulses ( $p < 0.05$ ). However, longer holding times between pulses (1 and 5 min) did not result in greater spore inactivation ( $p > 0.05$ ).

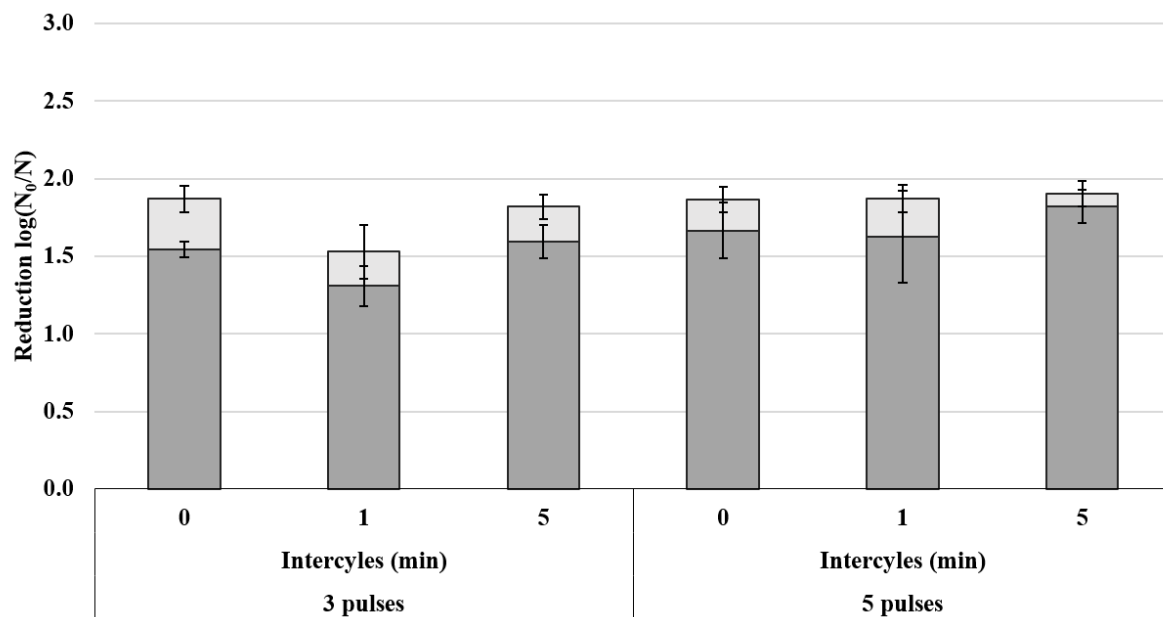
*Bacillus cereus* spores were also treated by pressure cycling at 400 MPa, at 60°C, with 3, 4, or 5 pulses of 5 min each, and with different times between pulses of 0, 1, and 5 min (**Fig. 7**). As for *B. subtilis* spore inactivation, longer holding times between pulses did not result in greater spore inactivation for the first treatment tested (3 pulses): between 4.8 and 5.1 log inactivation. For both treatments of 4 and 5 pulses, higher inactivation (5.8-5.9 log) was observed when the time between pulses applied was 5 min ( $p < 0.05$ ).

For both species, increasing the time between pulses did not seem to increase spore inactivation in this study (except for *B. cereus* spores, for longer treatments (4 or 5 pulses) and longer time between pulses (5 min)). Perhaps the time between HP pulses was not sufficient to allow greater germination ratios. In fact, a longer time (1 h) at atmospheric pressure has previously been reported to greatly enhance *B. subtilis* spore germination and inactivation ratios (Nguyen Thi Minh, Dantigny, Perrier-Cornet, & Gervais, 2010). In the latter study, maintaining spores at atmospheric pressure for 60 min between two pulses (350 MPa) allowed 4.5 log inactivation, whereas the absence of an intercycle led to an inactivation

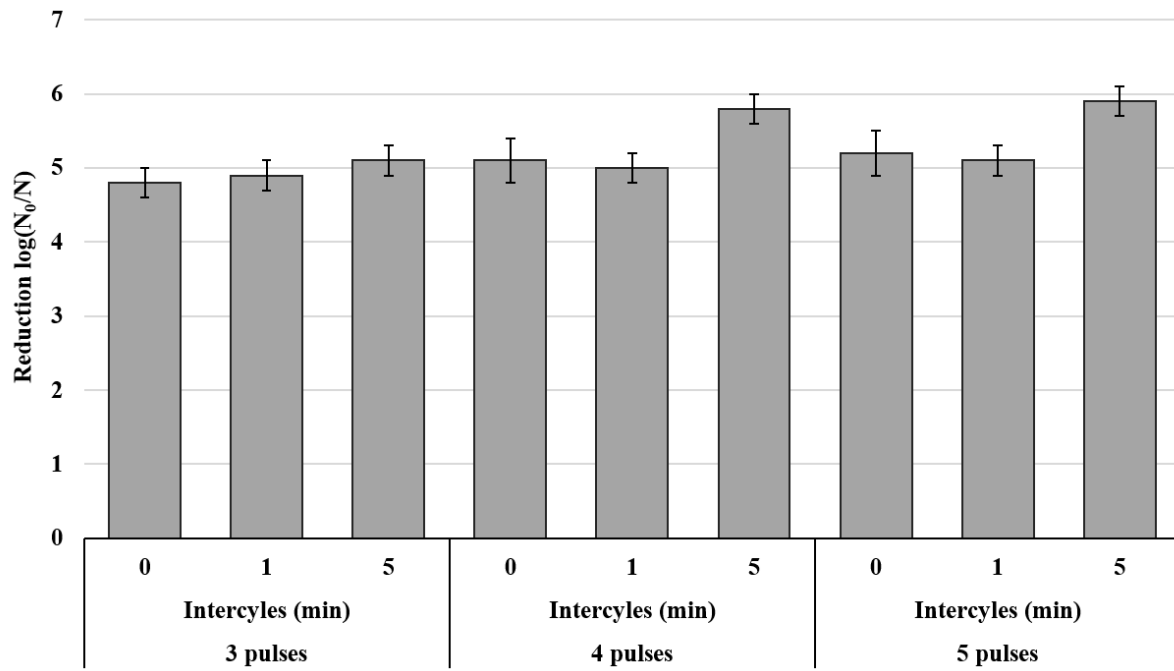


of only 2 log. Several studies have previously employed two HP treatments: the first at moderate pressure (100-400 MPa) and the second at higher pressure (> 500 MPa) with a prolonged period between the two treatments (30-60 min) to stimulate germination and enhance inactivation (Capellas, Mor-Mur, Gervilla, Yuste, & Guamis, 2000; Mills, Earnshaw, & Patterson, 1998; Wuytack, Boven, & Michiels, 1998). An extended time at atmospheric pressure (> 60 min) or a long time at moderate pressure (210 min at 60 MPa) (Capellas et al., 2000) could stimulate the transition from stage 1 to stage 2 of germination and therefore increase the inactivation of germinated spores in subsequent pressure treatment.

In our case, we increased the time between pulses by a few minutes to remain within reasonable industrial processing conditions. It would be necessary to increase the time between pulses to highlight this phenomenon.



**Figure 6:** Effect of time between pulses (intercycles) on germination and inactivation of pressure-treated *B. subtilis* spores. 3 or 5 pulses × 5 min, 350 MPa, 40°C. Bars: inactivation (dark gray bars) and germination (light gray bars) of spores treated by pressure cycling. Error bars represent SD calculated from independent triplicates.



**Figure 7:** Effect of time between pulses (intercycles) on inactivation of pressure-treated *B. cereus* spores. 3, 4, or 5 pulses × 5 min, 400 MPa, 60°C. Error bars represent SD calculated from independent triplicates.

### 3.4. Effect of compression and decompression rates on pressure cycling inactivation of *B. subtilis* spores

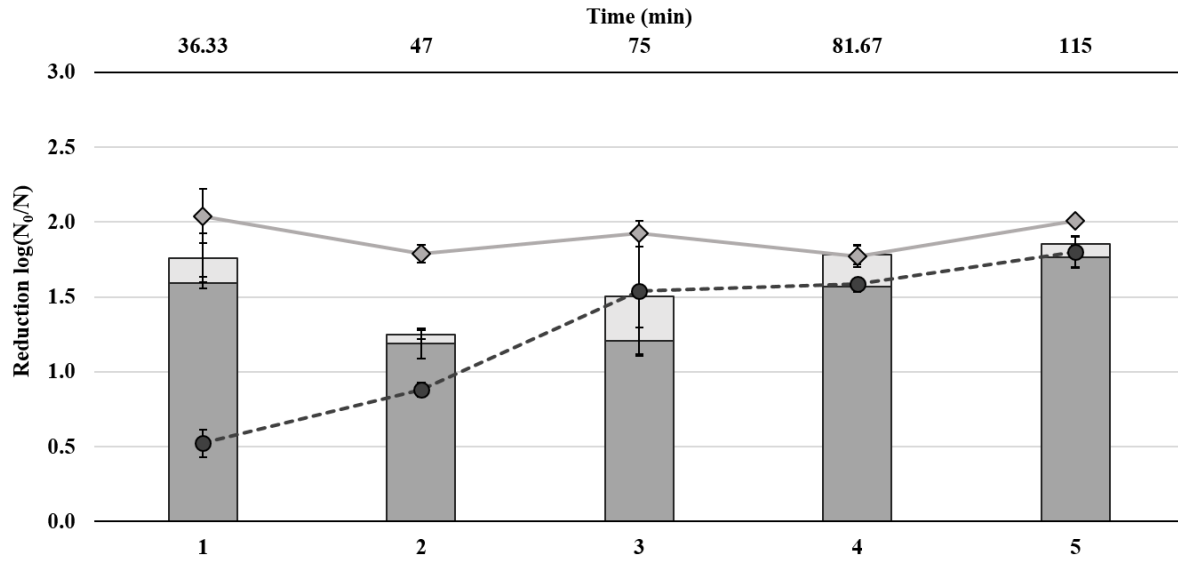
Germination and inactivation of *B. subtilis* spores were evaluated after pressure cycling (350 MPa, 40°C, 4 pulses of 5 min, with 5 min between pulses) with varying compression and decompression rates. Continuous HP treatments were also performed with the same variations in compression and decompression rates to evaluate the importance of these parameters on the cyclic effect.

The results presented in **Fig. 8** reveal a slight increase (approximately 0.4 log) in spore inactivation for slow pressure rates (kinetics 4-5) or the highest pressure rate tested (kinetics 1) for pressure cycling treatment. As expected for the continuous treatment, longer treatment (for slow kinetics) led to greater inactivation, whereas germination ratios remained almost constant. This longer time under pressure can also explain the greater inactivation ratios observed for slow cycling kinetics (4-5). Considering only the time under pressure, greater rates would give shorter time under pressure and thus lower inactivation, which is effectively observed except for kinetics 1. This compression rate effect requires a compression  $> 17.5$  MPa/s, that is, 350 MPa in 20 s. This pulse effect was only observed with cyclic treatment because control samples that were pressurized in the same manner exhibited only weak inactivation (0.52 log) related to the short treatment time (36 min).

The higher inactivation of spores with slow compression rates has already been observed in several studies: for *B. subtilis* at 600 MPa/60-80°C (maximum compression rate 15 MPa/s, (Syed et al., 2012)) or *B. amyloliquefaciens* at 600 MPa/60-105°C (maximum compression rate 18 MPa/s, (Ratphitagsanti et al., 2009)). It is generally postulated that a lower compression rate leads to a longer time under pressure, and hence, greater inactivation. Moreover, a slow compression rate allows stimulation of spore germination, which is enhanced at moderate pressure levels (between 80 and 200 MPa). Our results seem to confirm

these two hypotheses, with an increase in spore inactivation and an increase in germination ratio (inactivation after heat treatment) with slow compression and decompression.

The rapid compression effect on spore inactivation observed in this study could be related to the adiabatic compression heat, which is limited by rapid heat diffusion in the stainless-steel HP cylinder (in our case, 8°C). As mentioned in the Material and Methods section, only for this condition, adiabatic heating could not be limited to less than +3°C. In the literature, temperature increases of samples resulting from rapid compression are rarely recorded and depend mainly on experimental parameters (compression rate, pressurization medium, volume, etc.). In previous studies (Luu-Thi et al., 2015; Syed et al., 2012), specific experimental conditions led to a lower temperature immediately after compression for fast compression and thus with no fast compression inactivation effect on spores. Conversely, in changing compression medium characteristics, Robertson and co-workers (Robertson, Carroll, & Pearce, 2008) observed an increase in *Bacillus* spore inactivation with increasing adiabatic compression heat at the same compression rate. At the compression rate used (< 200 MPa/s), effects of compression kinetics are mainly due to adiabatic compression heat; higher compression or decompression rates are necessary to cause mechanical perturbations of spores, as previously observed with gas release (Colas de la Noue, Espinasse, Perrier-Cornet, & Gervais, 2012).



**Figure 8:** Inactivation of *B. subtilis* spores after pressure treatments using various compression and decompression rates. Curves: inactivation (black dashed curve) and germination (gray solid curve) of spores treated by continuous HP treatments. Bars: inactivation (dark gray bars) and germination (light gray bars) of spores treated by pressure cycling. 350 MPa, 40°C, 4 pulses of 5 min, with 5 min between pulses. **1:** CR = 17.5 MPa.s<sup>-1</sup>, DR = 350 MPa.s<sup>-1</sup>; **2:** CR = 2.5 MPa.s<sup>-1</sup>, DR = 8.75 MPa.s<sup>-1</sup>; **3:** CR = 1.17 MPa.s<sup>-1</sup>, DR = 1.17 MPa.s<sup>-1</sup>; **4:** CR = 1 MPa.s<sup>-1</sup>, DR = 1 MPa.s<sup>-1</sup>; **5:** CR = 0.58 MPa.s<sup>-1</sup>, DR = 0.58 MPa.s<sup>-1</sup>.

### 3.5. Modeling of *B. subtilis* spores germination and inactivation by HP

Inactivation kinetics models are commonly used to describe spore inactivation by HP treatment. In this study, inactivation kinetic modeling seems essential to compare the phenomena involved in spore inactivation between continuous and cycling HP treatments.

Different modeling approaches have been proposed in the literature for spore inactivation. For thermal treatments, a first-order kinetic model was used to model spore inactivation, giving rise to a linear function with the rate constant  $k$  as the slope. For HP treatments, spore inactivation depends on the kinetics of several successive processes, and thus requires models that are more complex. Reineke *et al.* (2012) applied a two-step kinetic model based on a simple differential chemical equation (dormant spore  $\rightarrow$  germinated spore  $\rightarrow$  inactivated spore) to develop a second-order model for high-pressure, high-temperature spore inactivation. It is commonly assumed that *Bacillus* spore germination occurs in two stages (Setlow, 2003). The transition from stage 1 to stage 2 of germination is mainly based on enzymatic reactions. In the first stage of germination, spores release DPA and become sensitive to heat. In the second stage, the external layers are degraded by CLE enzymes, allowing the rapid swelling of spores and their subsequent sensitization to other stresses (Nguyen Thi Minh *et al.*, 2010; Setlow, 2003). Here, we chose a three-step kinetic model to highlight the two stages of spore germination at the origin of spore inactivation. We further varied the model parameters according to pressure and pressure pulses.

Based on the literature, the following hypotheses concerning the pressure dependence of these rates were formulated:

- $k_1$  was assessed for pressure-induced spore germination. Because of the lack of data concerning germination kinetics at different pressures, germination kinetics was considered to be 0 at atmospheric pressure, increased linearly with pressure up to 200

MPa, and remained constant at pressures > 350 MPa. The  $k_1$  value at this interval (200-350 MPa) was optimized for this model.

- $k_2$  was attached to a transformation that is essentially governed by CLE enzymes. These enzymatic reactions may be modified under pressure. Pressure was not necessary for this transformation, but it could modulate its kinetics. A simple thermodynamic model is chosen as follows:

$$\frac{\partial \ln k_2}{\partial P} = -\frac{\Delta V}{RT}$$

Considering  $\frac{\Delta V}{RT}$  to be a constant. Thus, only two points are necessary. The  $k_2$  value at atmospheric pressure ( $4.7 \times 10^{-3} \text{ s}^{-1}$ ) was calculated from the literature (Pandey et al., 2013) and  $k_2$  at 350 MPa was obtained from optimization.

This parameter and its variations were adjusted during optimization, with a value at atmospheric pressure deduced from the literature.

- $k_3$  describes the inactivation of germinated spores. Stage 2-germinated spores became sensitive to lethal perturbations due to the increase in core water, similar to vegetative forms. Considering stage 2-germinated spores as vegetative bacteria,  $k_3$  was adapted from a model presented in the literature (Heinz & Knorr, 1996) and was not adjusted in this study.

In a first step, model optimization was performed on continuous HP treatments with variable durations (from 8 min to 4 h at 350 MPa and 40°C) in order to minimize the sum of squared deviations (SSD) between the model and experimental data (the optimal value of the criterion  $1.89 \times 10^7$  is relevant according to the population level and variations). The results displayed in **Fig. 9** show an accelerated decrease in dormant spores as well as a shoulder and tailing formation. As dormant spore numbers decreased, the maximum amount of spore germination was deduced from modeling, corresponding to stage 1-germinated spores. Pressure holding

did not allow stage 1-germinated spores to reach stage 2. Experimental data are also presented in this figure, corresponding to the dormant spores (NHPT<sub>exp</sub>) and inactivated spores (I<sub>exp</sub>). The resulting model correctly translated the evolution of D and I populations. It could also predict the evolution of the G1 and G2 populations, which have not been measured experimentally.

Then,  $k_1$  and  $k_2$  were calculated for each pressure variation over time. Optimal values of  $k_1$  and  $k_2$  were obtained, as shown in **Fig. 10**. A slight decrease in  $k_2$  with pressure was observed, supporting an increase in reaction  $\Delta V$ , which is consistent with the hydrolysis reaction. The  $k_1$  value at 350 MPa is also consistent with the kinetics of DPA leakage measured at the same pressure and temperature ( $4.6 \times 10^{-3} \text{ s}^{-1}$  at 300 MPa at 40°C for *B. subtilis* PS832) (Reineke et al., 2012).

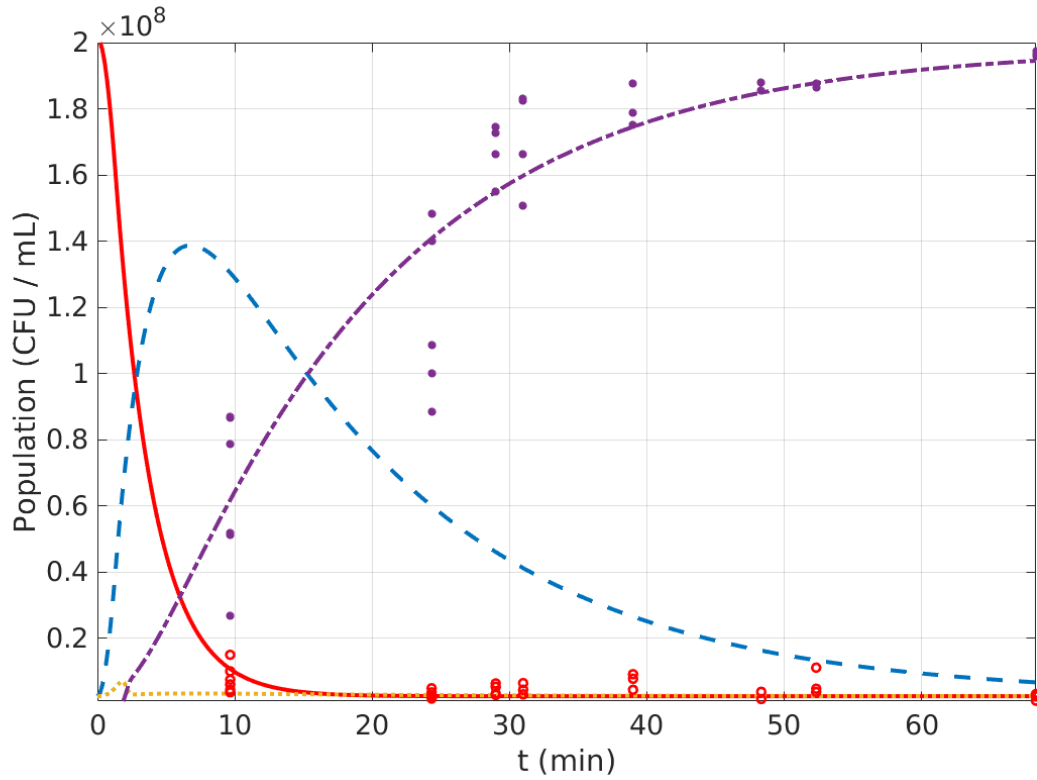
For HP treatment at moderate temperatures, some spores remain resistant regardless of the time employed. It is well known that some spores which are called “super dormant” (SD) are not able to germinate with nutrients, probably because they have very low GR levels (Stewart & Setlow, 2013). High-temperature treatment or chemical germination is generally necessary to induce germination. A relationship between SD spores in nutrients, presence of GR, and resistance to moderately high pressure exists but is largely dependent on GR (Doona et al., 2014). Similarly, high pressure/moderate temperature-resistant spores could be termed as HPSD (Delbrück, Zhang, Hug, et al., 2021b). To fit the normalized experimental data, the HPSD values needed to be adjusted. The optimized HPSD level ( $2.10 \times 10^6$  spores/mL) was consistent with the experimentally obtained HPSD value ( $4.98 \times 10^6$  spores/mL) from experiments with a long holding time at a given pressure and temperature level.

Finally, using these rates, it was possible to stimulate spore germination and inactivation by HP cycling treatment (**Fig. 11**). The previous model allows simulation by solving the ODEs

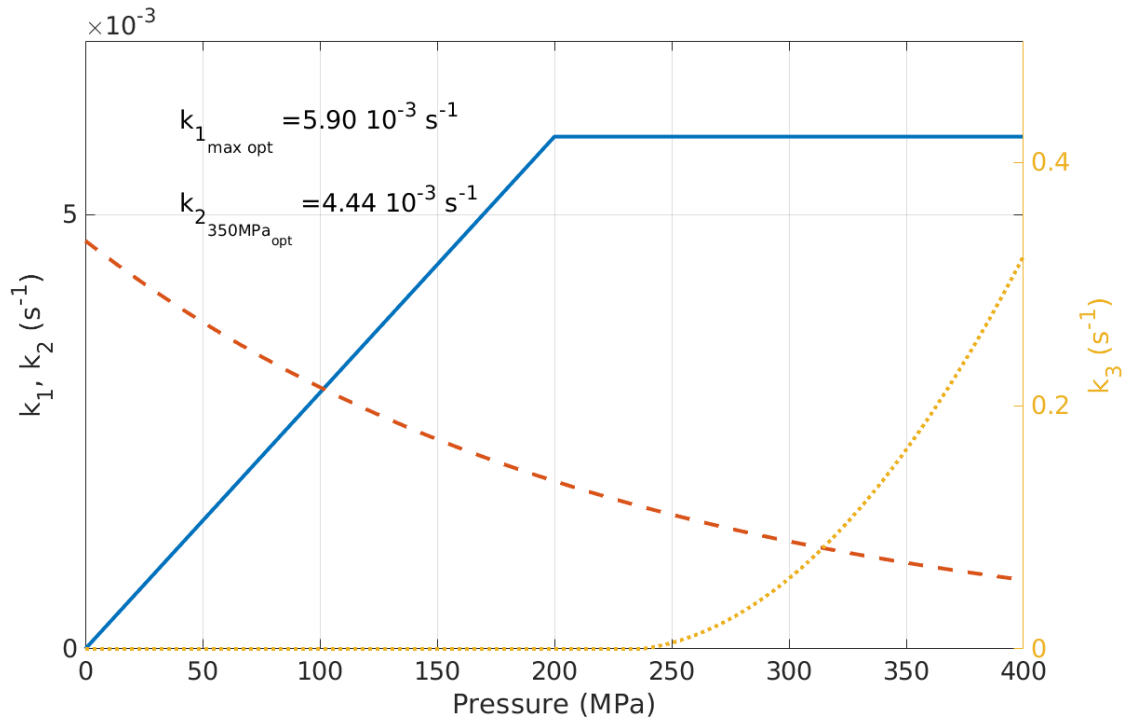


for all experimental conditions: pressure variation regardless of the number of cycles, time between pulses, and CR and DR variations. **Fig. 11** shows that the experimental data fit well with the predicted data, which supports our hypotheses regarding the mechanisms of inactivation under HP cycling. Germination induction was maximal within the first pulse. An increase in the number of pulses had no apparent effect on germination induction. The amount of stage 2-germinated spores was slightly higher for the first two times between pulses, indicating that CLEs may be inactive under HP. For the subsequent times between pulses, the abundance of stage 2-germinated spores decreased, perhaps due to the low level of germination induction (stage 1-germinated spores).

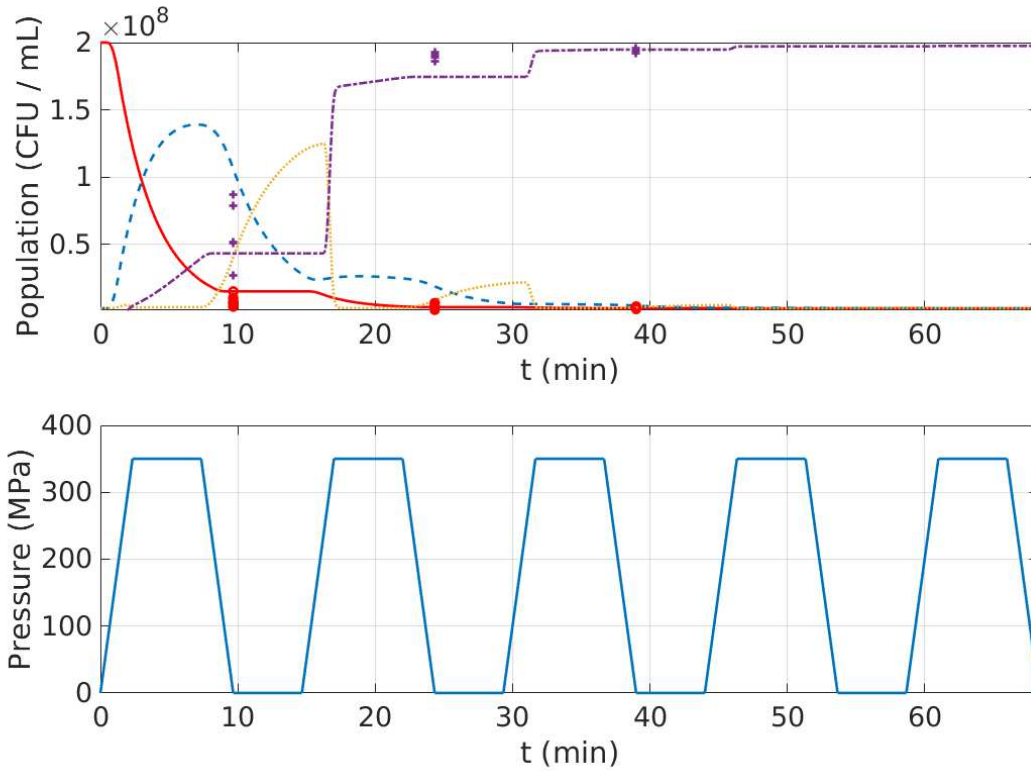
The fact that the model does not fit well for the greatest compression rate experimental data is consistent with the dependence of the model parameters on temperature elevation during such treatment. In fact, adiabatic heating is responsible for a deviation of +8°C under such conditions, whereas  $k_1$ ,  $k_2$ ,  $k_3$ , and HPSD were optimized at 40°C only.



**Figure 9:** Evolution of the different stages of *B. subtilis* spore germination and inactivation as a function of holding time under continuous HP treatment (350 MPa, 40°C). Solid red curve represents calculated dormant spores (D); dashed blue curve represents calculated stage 1-germinated spores (G1); dotted yellow curve represents calculated stage 2-germinated spores (G2); and dash-dotted purple curve represents calculated inactivated spores (I). Solid purple dots represent experimentally inactivated spores ( $I_{exp}$ ); empty red dots represent experimentally HPHT treated spores ( $NHPT_{exp}$ ).



**Figure 10:** Rate fitting for continuous HP treatments of *B. subtilis* spores ( $k_1$ ,  $k_2$  were obtained by optimization whereas  $k_3$  was adapted from (Heinz & Knorr, 1996), in  $\text{s}^{-1}$ ). Solid blue curve, dashed orange curve and dotted yellow curve represent  $k_1$ ,  $k_2$ , and  $k_3$  calculated rates, respectively.



**Figure 11:** Evolution of the different stages of *B. subtilis* spore germination and inactivation as a function of pulses number (350 MPa, 40°C). Solid red curve represents calculated dormant spores (D); dashed blue curve represents calculated stage 1-germinated spores (G1); dotted yellow curve represents calculated stage 2-germinated spores (G2); and dash-dotted purple curve represents calculated inactivated spores (I). Purple dots represent experimentally inactivated spores (I<sub>exp</sub>); red points represent experimentally HPHT-treated spores (NHPT<sub>exp</sub>).

#### 4. Conclusions

In this study, inactivation of *B. cereus* and *B. subtilis* spores was measured after HP cycling under varying conditions and compared with equivalent continuous HP treatments. We observed that inactivation was mainly related to the pressure holding time. HP cycling had only a marginal effect on inactivation, probably due to germination acceleration in the first cycles and adiabatic heating for rapid compression rates. The design of a three-step kinetics model supports our hypothesis regarding the mechanisms involved in spore inactivation. Further investigations focusing on temperature implementation in the model could allow a better understanding of spore germination and inactivation mechanisms. In the same way, further experiments involving quantification of the released DPA and observation of cortex lysis by CLEs could lead to a comprehensive physiological characterization of HP-treated spores. Finally, further studies focusing on a better understanding of HPSD spores and their variations in pressure and temperature are relevant in order to improve spore inactivation by HP treatment.

From an industrial point of view, the cycling treatment does not seem to reduce treatment duration while inducing a quicker fatigue of the HP equipment. Moreover, commercial HP applications available currently work at ambient temperature.

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#### Conflicts of interest

None of the authors declares a conflict of interest.

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## Figure Captions

**Figure 1:** Characteristics of cycling and continuous HP treatments. Pressure recording is plotted as a black line and temperature as a gray one.

**Figure 2:** Inactivation model based on the spore germination sequence wherein  $D + \text{HPSD}$  represents the initial population composed of dormant (D) and superdormant spores (HPSD), G1 represents germinated spores at stage 1, G2 germinated spores at stage 2, and I inactivated spores by the treatment.  $k_1$ ,  $k_2$  and  $k_3$  are model parameters ( $\text{s}^{-1}$ ).

**Figure 3:** Inactivation of *B. cereus* spores in a suspension of infant milk after pressure treatments. Bar: 4 pulses  $\times$  5 min, 350 MPa, 38°C, with a time between pulses of 5 min. Dashed curve: continuous HP treatment for 5, 20, and 35 min. Error bars represent SD calculated from independent triplicates.

**Figure 4:** Effect of pulse number (1, 2, 3 or 5) on germination and inactivation of pressure treated *B. subtilis* spores. *Bacillus subtilis* spores were suspended in MES buffer solution and treated at 350 MPa, 40°C, pulse holding time of 5 min, and time between pulses of 5 min. Curves: inactivation (black dashed curve) and germination (gray solid curve) of continuous HP treatments. Bars: inactivation (dark gray bars) and germination (light gray bars) of spores treated by pressure cycling. Error bars represent SD calculated from independent triplicates; (\*) indicate statistically significant difference ( $p < 0.05$ ) between the equivalent continuous and cycling HP treatments.

**Figure 5:** Effect of pulse number (1, 2, 3, 4 or 5) on inactivation of pressure treated *B. cereus* spores. The black dashed curve represents inactivation of spores treated by continuous HP treatments. Gray columns represent inactivation of spores treated by pressure cycling. *Bacillus cereus* spores were suspended in TS buffer solution and treated at 300 MPa, 50 or

60°C, pulse holding time of 5 min, time between pulses of 1 min. Error bars represent SD calculated from independent triplicates.

**Figure 6:** Effect of time between pulses (intercycles) on germination and inactivation of pressure-treated *B. subtilis* spores. 3 or 5 pulses × 5 min, 350 MPa, 40°C. Bars: inactivation (dark gray bars) and germination (light gray bars) of spores treated by pressure cycling. Error bars represent SD calculated from independent triplicates.

**Figure 7:** Effect of time between pulses (intercycles) on inactivation of pressure-treated *B. cereus* spores. 3, 4, or 5 pulses × 5 min, 400 MPa, 60°C. Error bars represent SD calculated from independent triplicates.

**Figure 8:** Inactivation of *B. subtilis* spores after pressure treatments using various compression and decompression rates. Curves: inactivation (black dashed curve) and germination (gray solid curve) of spores treated by continuous HP treatments. Bars: inactivation (dark gray bars) and germination (light gray bars) of spores treated by pressure cycling. 350 MPa, 40°C, 4 pulses of 5 min, with 5 min between pulses. **1**: CR = 17.5 MPa.s<sup>-1</sup>, DR = 350 MPa.s<sup>-1</sup>; **2**: CR = 2.5 MPa.s<sup>-1</sup>, DR = 8.75 MPa.s<sup>-1</sup>; **3**: CR = 1.17 MPa.s<sup>-1</sup>, DR = 1.17 MPa.s<sup>-1</sup>; **4**: CR = 1 MPa.s<sup>-1</sup>, DR = 1 MPa.s<sup>-1</sup>; **5**: CR = 0.58 MPa.s<sup>-1</sup>, DR = 0.58 MPa.s<sup>-1</sup>.

**Figure 9:** Evolution of the different stages of spore germination and inactivation as a function of holding time under continuous HP treatment (350 MPa, 40°C). Solid red curve represents calculated dormant spores (D); dashed blue curve represents calculated stage 1-germinated spores (G1); dotted yellow curve represents calculated stage 2-germinated spores (G2); and dash-dotted purple curve represents calculated inactivated spores (I). Solid purple dots represent experimentally inactivated spores (I<sub>exp</sub>); empty red dots represent experimentally HPHT treated spores (NHPT<sub>exp</sub>).

**Figure 10:** Rate fitting for continuous HP treatments of *B. subtilis* spores ( $k_1$ ,  $k_2$  were obtained by optimization whereas  $k_3$  was adapted from (Heinz & Knorr, 1996), in  $s^{-1}$ ). Solid blue curve, dashed orange curve and dotted yellow curve represent  $k_1$ ,  $k_2$ , and  $k_3$  calculated rates, respectively.

**Figure 11:** Evolution of the different stages of *B. subtilis* spore germination and inactivation as a function of pulses number (350 MPa, 40°C). Solid red curve represents calculated dormant spores (D); dashed blue curve represents calculated stage 1-germinated spores (G1); dotted yellow curve represents calculated stage 2-germinated spores (G2); and dash-dotted purple curve represents calculated inactivated spores (I). Purple dots represent experimentally inactivated spores ( $I_{exp}$ ); red points represent experimentally HPHT-treated spores (NHPT<sub>exp</sub>).