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ABSTRACT

Bacillus sporothermodurans is a milk spoilage bacterium producing resistant endospores that survive ultra-high temperature treatment. The inactivation of *B. sporothermodurans* LTIS27 spores by combined hydrostatic pressure and heat treatments was studied. A central composite experimental design was used to evaluate the effect of pressure (300–500 MPa), temperature (30–50 °C), and pressure-holding time (10–30 min) on the inactivation of spores in distilled water and skim milk. The inactivation observed was shown to fit well with the values predicted by the quadratic equation, since R^2_{adj} were 0.970 and 0.977 in distilled water and milk, respectively. By analyzing the response surface plots, the inactivation was shown to be higher in distilled water than in milk under all the conditions tested. This was probably due to a protective effect of milk against inactivation by pressure. The optimum process parameter values for a 5-log cycle reduction of spores were calculated as 477 MPa/48 °C for 26 min and 495 MPa/49 °C for 30 min in water and in milk, respectively. This study shows the efficiency of hydrostatic pressure in combination with moderate temperature to inactivate *B. sporothermodurans* spores. Such treatments could be applied by the dairy industry to ensure the commercial sterility of UHT milk.

1. Introduction

Aerobic spore-forming bacteria of the genus *Bacillus* are often present in raw milk and play an important role in the spoilage of milk and milk products (Crielly, Logan, & Anderton, 1994). The use of ultrahigh-temperature (UHT) processing should inactivate the spores of *Bacillus* species and result in fluid milk products with a long shelf-life without refrigeration. However, defects in UHT milk product stability have been reported (Hammer, Lembke, Suhren, & Heeschen, 1995; Klijn et al., 1997). This nonstability appeared to be caused by the presence of highly heat-resistant bacterial endospores (HRS). HRS were first detected in UHT milk from southern Europe in 1985 and were later identified in other countries both in and outside Europe (Hammer et al., 1995). They were found to belong to the species *Bacillussporothermodurans*, first described by Pettersson, Lembke, Hammer, Stackebrand, and Fergus (1996). It thus became necessary to develop more efficient processes to inactivate HRS completely and ensure milk commercial sterility.

The combination of heat with high hydrostatic pressure (HP) has been proposed as an interesting approach for the inactivation of bacterial spores in food (Heinz & Buckow, 2010). HP has been shown to be efficient at destroying vegetative bacteria, and yeasts although bacterial spores appear to be more resistant to HP (Nakayama, Yano, Kobayashi, Ishikawa, & Sakai, 1996). San Martin, Barbosa-Canovas, and Swanson (2002) have shown that very high pressure levels (1000 MPa), at ambient temperature, are not effective in inactivating bacterial spores; however, this can be achieved by combining HP with temperatures higher than 40 °C (Ju, Gao, Yao, & Qian, 2008; Kalchayanand, Dunne, Sikes, & Ray, 2004; Oh & Moon, 2003). Previous studies have concluded that

Abbreviations: DPA, Dipicolinic acid; HP, High pressure; HRS, Highly heat-resistant spores; RSM, Response surface methodology; UHT, Ultra-high temperature.

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a combination of pressure and moderate heat is always required to inactivate effectively spores of *Bacillus* species such as *Bacillus anthracis* (Cléry-Barraud, Gaubert, Masson, & Vidal, 2004), *Bacillus cereus* (Kannapha et al., 2008; Opstal, Bagamboula, Vanmuysen, Wuytack, & Michiels, 2004), *Bacillus subtilis* (Gao, Ju, & Jiang, 2006; Minh, Dantigny, Perrier-Cornet, & Gervais, 2010) and even *Clostridium* species (Kalchayanand et al., 2004; Mills, Earnshaw, & Patterson, 1998). For example, the inactivation rate of *B. anthracis* spores was shown to be higher (4.4-log cycle reduction) after a treatment at 280 MPa and 45 °C than after a treatment at 500 MPa and 20 °C (1-log cycle reduction) (Cléry-Barraud et al., 2004). Mills et al. (1998) showed that *Clostridium sporogenes* was resistant to HP, with treatments of 600 MPa for 30 min at 20 °C causing no significant inactivation, while the combination of mild heat (60 °C) with pressure (600 MPa for 30 min) resulted in a 3-log cycle reduction in spore count.

The mechanisms of spore-killing by heat, irradiation and chemicals have been well studied and described but those using HP are still poorly understood. It is now well known that HP can induce spore germination (Margosch, Ganzle, Ehrmann, & Vogel, 2004; Paidhungat et al., 2002; Wuytack, Boven, & Michiels, 1998; Wuytack, Soons, Poschet, & Michiels, 2000). Pressure (100–600 MPa) at 20–40 °C induces the opening of channels for the release of dipicolinic acid (DPA) from the spore core, which leads to spore germination (Paidhungat et al., 2002; Wuytack et al., 2000). Margosch et al. (2004) treated mashed carrots inoculated with spores of *B. subtilis*, *Bacillus amyloliquefaciens* and *Bacillus licheniformis* with HP and heat (200–800 MPa and 60–80 °C). Their results confirm that, at a pressure between 600 and 800 MPa and a temperature higher than 60 °C, DPA-free spores are inactivated by moderate heat independently of the pressure level.

The aim of this study was to evaluate the effectiveness of HP treatment, alone and in association with heat, on the inactivation of *B. sporothermodurans* spores in distilled water and in skim milk. For this purpose, response surface methodology (RSM) was used to describe spore inactivation in terms of pressure, temperature, and pressure-holding time.

2. Materials and methods

2.1. Bacterial strain

B. sporothermodurans strain LTIS27 was isolated from some contaminated samples of UHT-milk produced in Tunisia using the method previously described (Klijn et al., 1997). This strain was identified by PCR method using primers BSPO-F2 (5′-ACGGCT CAACCGTGGAG-3′) and BSPO-R2 (5′-GTAACCTCGCGGTCTA-3′) (Biogene), as described by Scheldeman, Herman, Goris, De Voe, and Hendrickx (2002). The identification was confirmed by sequencing the 16S rRNA gene after PCR amplification as described by Winker and Woese (1991). *B. sporothermodurans* LTIS27 presents a high degree of similitude (99.8%) with the reference strain M215T type II regarding 16S rRNA gene sequences (Aouadhi et al., 2012), a specific strain of the UHT milk and named HRS clone (Montanari et al., 2004).

B. sporothermodurans LTIS27 was grown on brain–heart infusion agar supplemented with 1 mg/l vitamin B_{12} (Sigma–Aldrich) (BHI-B_{12}) at 37 °C. Twenty-four hours before use, cultures were held in BHI-B_{12} broth on a rotary shaker at 37 °C to obtain a working culture.

2.2. Spore preparation

Spores were prepared according to the modified method of Ireland and Hanna (2002). A working culture (18 h, 37 °C under agitation) was diluted 1:10 into sporulation medium (25 g l^{-1} nutrient broth, 1 mg l^{-1} vitamin B_{12}, 8 mg l^{-1} $MnSO_4 \cdot H_2O$, and 1 g l^{-1} $CaC1_2 \cdot H_2O$) then incubated for 7 days at 37 °C. This medium was then centrifuged at 6000 *g* for 10 min. The resulting pellet, containing spores and non-sporulated vegetative cells, was washed three times with sterile distilled water, resuspended in 5 ml of sterile distilled water and treated at 100 °C for 30 min to kill the vegetative cells. The endospore suspension was centrifuged and washed 4 times as described above. The cleaned endospore preparation was stored at a final concentration of approximately 10^7–10^8 spores ml^{-1} in distilled water at 4 °C.

Table 1
Codes and levels of factors chosen for the trials.

	Symbols		Level[a]				
	Coded	Uncoded	−1.68	−1.0	0.0	1.0	1.68
Pressure (MPa)	x_1	X_1	231.8	300	400	500	568.2
Temperature (°C)	x_2	X_2	23.8	30	40	50	56.2
Pressure-holding time (min)	x_3	X_3	3.2	10	20	30	36.8

[a] $x_1 = (X_1 - 400)/100$; $x_2 = (X_2 - 40)/10$; $x_3 = (X_3 - 20)/10$.

2.3. High hydrostatic-pressure treatment

High-pressure processing was carried out in a 3-l vessel (ACB Pressure Systems, Nantes, France) equipped with temperature and pressure regulatory devices, capable of achieving a maximum pressure of 600 MPa and a temperature of up to 60 °C. The samples (2 ml) were diluted 1:2 in sterile distilled water or in skim milk (5×10^7 spores ml^{-1}), enclosed in polyethylene pouches, and subjected to the combined pressure and heat treatment protocols given in Table 2. The compression rate was 34 Pa/s and the pressure release was almost instantaneous. The temperature of the water in the vessel was maintained by circulating water within the double wall of the device from a water bath. The temperature increase due to the adiabatic heating effect was equivalent to approximately 1–3 °C/100 MPa depending on the initial temperature of the water in the vessel. After treatment, the pouches were immediately placed on ice and analyzed within 10 min.

2.4. Enumeration of surviving spores

Serial dilutions of treated samples were prepared in peptone water (0.01%) and the viable counts were determined by plating

Table 2
Central composite design arrangement for study the inactivation of B. *sporothermodurans* LTIS27 by pressure and moderate temperature.

Trial no.	Factors		
	X_1 (MPa)	X_2 (°C)	X_3 (min)
1	568.2	40.0	20.0
2	500.0	50.0	10.0
3	400.0	40.0	36.8
4	300.0	50.0	30.0
5	300.0	50.0	10.0
6	400.0	40.0	20.0
7	231.8	40.0	20.0
8	500.0	30.0	10.0
9	400.0	23.8	20.0
10	500.0	30.0	30.0
11	500.0	50.0	30.0
12	300.0	30.0	10.0
13	300.0	30.0	30.0
14	400.0	40.0	20.0
15	400.0	40.0	3.2
16	400.0	40.0	20.0
17	400.0	56.2	20.0

on BHI-B_{12} agar. The plates were incubated at 37 °C for 24 h then the colonies were enumerated. Inactivation was expressed as a logarithmic viability reduction, log (N_0/N), with N and N_0 the colony counts after treatment and in the untreated sample, respectively.

2.5. Experimental design

A rotatable central composite experimental design with three independent factors ($\alpha = 1.68$) was used to study the inactivation of *B. sporothermodurans* spores in distilled water and in skim milk. The factors investigated were pressure, initial temperature in the vessel, and pressure-holding time. Each factor at five coded levels (indicated as −1.68, −1.0, 0.0, +1.0, +1.68 as shown in Table 1) were designed as experimental runs using the software STATGRAPHICS Centurion XV version 15.2.06. The real values of the independent variables (X) were coded according to equation: $x_i = (X_i - X_0)/\Delta X$, Where X is the real value of the independent variable; x_i is the coded value of the independent variable given by the experimental matrix; X_0 is the real value of the central point, and ΔX is the step change. X and x values are shown in Table 1. The experimental response was the number of log cycle reduction of *B. sporothermodurans* spores (log (N_0/N)), which was estimated, taking into account the influence of the experimental factors. Three experiments were performed at the center of the experimental domain in order to estimate the residual variance value. An analysis of variance and an estimation of response surface by multiple linear regressions were performed using the software Statgraphics Centurion XV version 15.2.06.

3. Results

3.1. Predictive response model

Inactivation of *B. sporothermodurans* LTIS27 spores by the combined effects of pressure, temperature, and pressure-holding time in distilled water and in skim milk was studied, using RSM. The response (Y) was measured in terms of log-cycle reduction (log N_0/N) (Y_1: in distilled water and Y_2: in skim milk). RSM is an empirical modeling technique used to estimate the relationship between the response variable and the test variable under consideration. This methodology has been used by some authors to evaluate the effect of various factors such as pressure–temperature on the inactivation of *B. cereus* (Ju et al., 2008) and *B. subtilis* (Gao & Jiang, 2005) spores. The response was represented by the following second-order polynomial equation, containing 10 estimated coefficients, where x_1 is pressure, x_2 is temperature, and x_3 is pressure-holding time.

$$\begin{aligned} Y1 = {} & 15.69 - 0.052x_1 - 0.296x_2 - 0.159x_3 + 0.00004x_1^2 \\ & + 0.0002x_1x_3 + 0.0004x_1x_2 + 0.002x_2^2 - 0.0003x_2x_3 \\ & + 0.002x_3^2 \end{aligned} \quad (1)$$

$$\begin{aligned} Y2 = {} & 9.82 - 0.033x_1 - 0.14x_2 - 0.149x_3 + 0.00003x_1^2 \\ & + 0.0002x_1x_3 + 0.0002x_1x_2 + 0.0016x_2^2 - 0.0001x_2x_3 \\ & + 0.002x_3^2 \end{aligned} \quad (2)$$

The analysis of variance (ANOVA) of the quadratic model was performed with Statgraphics. The R^2 values were 0.970 and 0.977 for Eqs. (1) and (2), respectively, indicating that the derived model fitted the experimental data. The statistical significance of each coefficient value was determined using the Fisher F-test. The model F-value is the ratio of the mean square regression to the mean square residual. For the two Eqs. (1) and (2), the coefficient values were calculated and tested for their significance. The P values were used as a tool to check the significance of each coefficient which, in turn, may indicate the pattern of interactions between variables. The linear coefficients (x_1, x_2, and x_3), the quadratic term coefficients (x_1^2, x_2^2 and x_3^2) and the cross coefficients (x_1x_2 and x_1x_3) were significant with small P values ($P < 0.05$). The other term coefficient (x_2x_3) was not significant.

3.2. Validation of the model

In order to validate the adequacy of polynomial equations (Eqs. (1) and (2)), ten experimental checks were carried out under different HP combinations of process parameters (Table 3). The experimental values were found to be significantly in agreement with the predicted values with a correlation coefficient (R^2) of 0.99 and a statistical significance level of $P < 0.0001$. Therefore, the model was proven to be quite reliable for predicting the inactivation of *B. sporothermodurans* spores in distilled water and in skim milk for treatment parameters in the range of 231.8–568.2 MPa, 23.8–56.2 °C and 3.2–36.8 min.

3.3. Response surfaces

Spores of *B. sporothermodurans* are known to be extremely resistant to heat treatment. The maximal load of this bacteria reported in stored milk is 10^5 cfu ml^{-1} of milk after 5 days of storage at 30 °C (Hammer et al., 1995; Klijn et al., 1997). Thus, the process parameters for a 5-log cycle reduction of *B. sporothermodurans* spores in distilled water and in skim milk were calculated using the model. The graphical representations were obtained by solving the regression Eqs. (1) and (2) using Statgraphics. In Figs. 1–3, response surfaces and contour plots show the effect of pressure, temperature and pressure-holding time on the inactivation of spores. In each figure, one factor is maintained constant at its optimal value determined by Statgraphics. Fig. 1 shows the effect of pressure and temperature while keeping the pressure-holding time at its optimal value (26 min in distilled water and 30 min in skim milk). The reduction of *B. sporothermodurans* spores increases with increasing pressure and temperature, reaching a 5-log cycle reduction at pressures of 460–500 MPa and temperatures of 45–50 °C.

The interaction of pressure and pressure-holding time at a constant temperature (48 °C in distilled water and 49 °C in skim milk) is shown in Fig. 2. In the pressure range of 480–500 MPa and

Table 3
Experimental and predicted results for ten check-points of inactivation of *B. sporothermodurans* LTIS27 spores in distilled water and in milk.

Trial no.	Factors			$Y = \log N_0/N$			
	X_1 (MPa)	X_2 (°C)	X_3 (min)	In distilled water		In skim milk	
				Observed	Predicted	Observed	Predicted
1	320	40	10	1.9	1.8	1.6	1.5
2	350	50	15	2.8	2.7	2.4	2.4
3	370	30	10	1.1	1.2	0.9	0.8
4	390	45	20	2.6	2.5	2.3	2.2
5	420	40	30	2.9	2.9	2.4	2.5
6	450	35	23	2.3	2.2	1.9	1.8
7	490	45	16	3.2	3.3	2.8	2.9
8	520	40	18	3.3	3.2	2.8	2.7
9	523	47	30	5.2	5.2	4.6	4.7
10	530	48	30	5.4	5.5	5.0	5.0

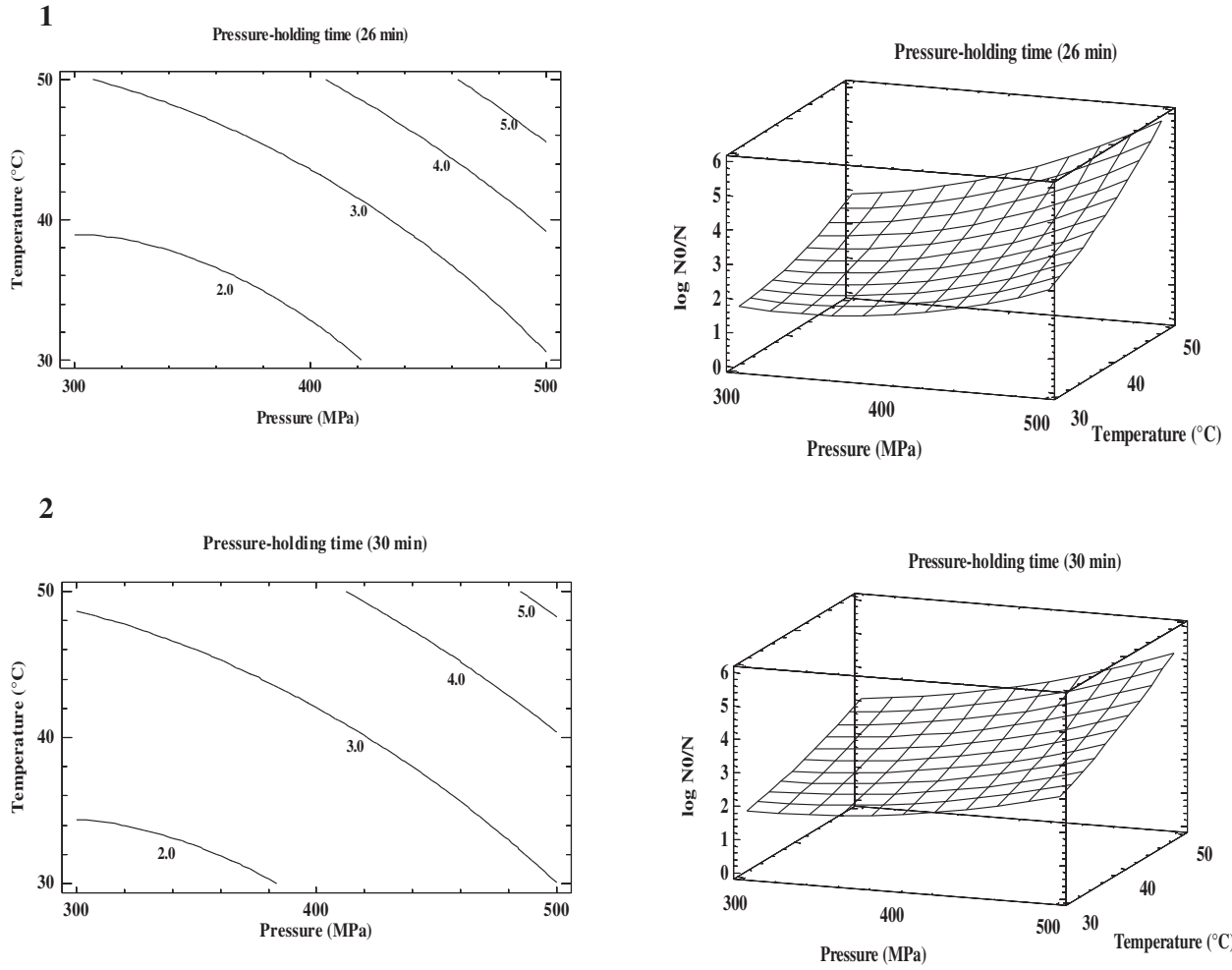

Fig. 1. Response surface and contour plots showing the effects of pressure and temperature on the inactivation of *B. sporothermodurans* LTIS27 spores. The data on the contour maps are log N_0/N in distilled water (1) and milk (2). N_0 represents the plate count of the untreated spore suspension (5×10^7 spore ml^{-1}) and N represents the plate count after pressure treatment.

the pressure-holding time range of 25–30 min, a reduction of 5-log cycle of *B. sporothermodurans* spores was calculated. The reduction of *B. sporothermodurans* spores as a function of temperature and pressure-holding time at a fixed pressure of 477 MPa in distilled water and 495 MPa in skim milk is given by the contour plots in Fig. 3. The reduction of 5-log cycle of *B. sporothermodurans* spores was obtained in the temperature range of 47–50 °C and in the pressure-holding time range of 24–30 min.

Optimum values of each parameter given by the model are as follows: 477 MPa/48 °C for 26 min in distilled water and 495 MPa/49 °C for 30 min in skim milk.

4. Discussion

The inactivation of *B. sporothermodurans* spores by combined HP and heat treatment was more efficient in distilled water than in skim milk. The process conditions leading to a 5-log cycle reduction of *B. sporothermodurans* spores were calculated using the model and less drastic conditions were obtained in water (477 MPa/48 °C for 26 min) than in milk (495 MPa/49 °C for 30 min). Some constituents of milk may protect spores against HP. The protective effect of food constituents on vegetative bacterial cells is well known and documented. Several authors have reported that bacteria are more resistant in a complex matrix such as milk or meat (Black, Huppertz, Fitzgerald, & Kelly, 2007; Chen & Hoover, 2003; Kannapha et al., 2008). Chen and Hoover (2003) demonstrated a strong baroprotective effect of whole UHT milk on *Yersinia enterocolitica*, with inactivation levels 3.5–4.5-log cycle lower than in phosphate buffer when treated at 350–450 MPa/22 °C for 10 min Black et al. (2007) studied the baroprotective effect of milk constituents on *Listeria innocua* treated by HP and stated that the divalent cations, calcium and magnesium, may protect cell membranes against HP. The effect of the food matrix on spore inactivation is more contradictory in the literature. While Kannapha et al. (2008) showed that crab meat had a protective effect against inactivation of *B. cereus* spores in comparison with spores suspended in distilled water treated at 550 MPa/40 °C for 15 min, Opstal et al. (2004) reported that the inactivation of *B. cereus* spores was 1-log cycle reduction higher in milk than in phosphate buffer after a treatment at 600 MPa, 60 °C for 10 min. The inactivation level of spores does not always follow the trend of germination since this was shown to be enhanced in crab meat (Kannapha et al., 2008) and in milk (Aouadhi et al., 2012; Opstal et al., 2004) compared to distilled water or buffer. The increase in germination in milk or in crab meat was attributed to the presence of some germinant, such as L-alanine, required in the germination process in addition to pressure. Thus, it seems that food nutrients have two opposite effects on spores under pressure; some may

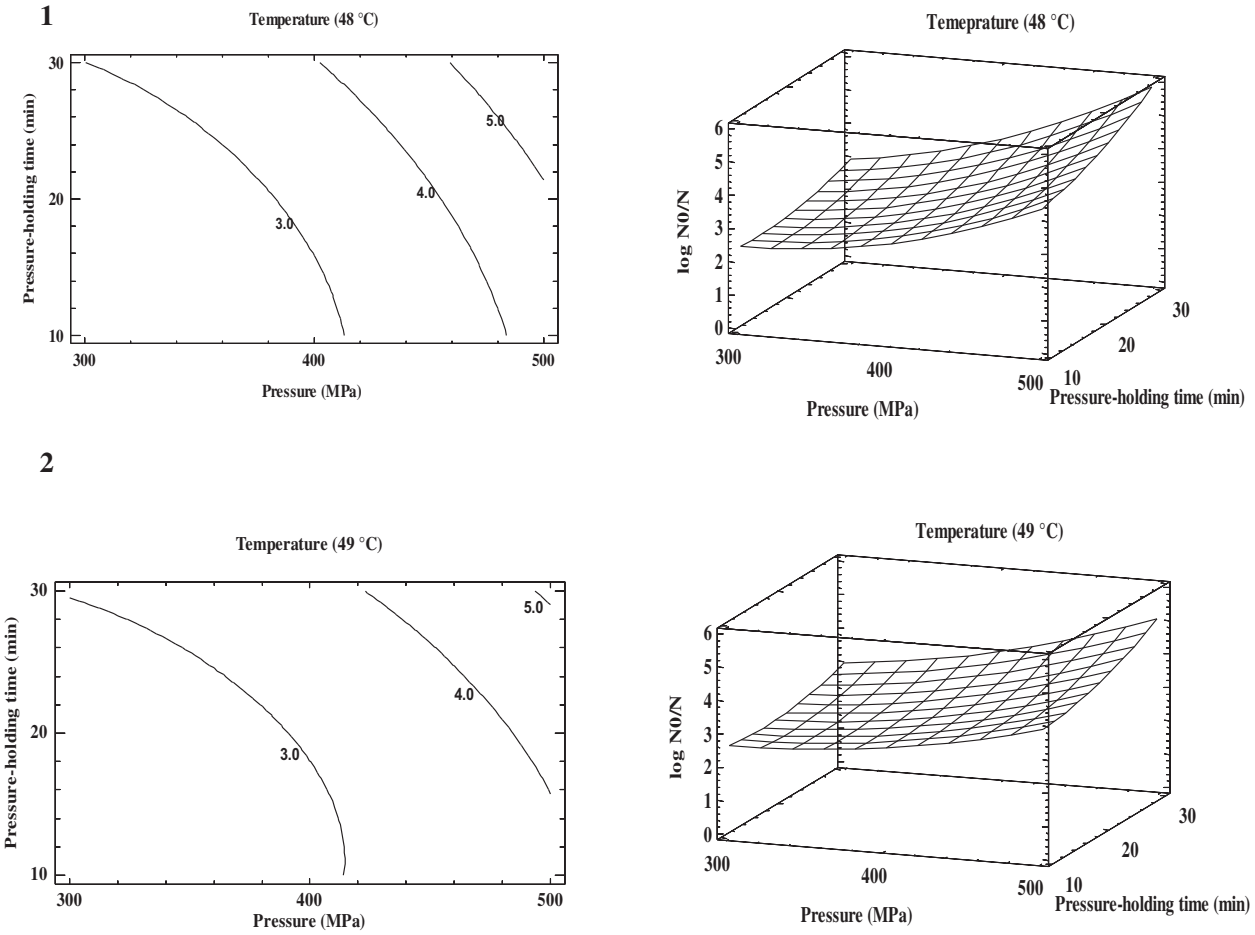

Fig. 2. Response surface and contour plots showing the effects of pressure and pressure-holding time on the inactivation of *B. sporothermodurans* LTIS27 spores. The data on the contour maps are log N_0/N in distilled water (1) and milk (2). N_0 represents the plate count of the untreated spore suspension (5×10^7 spore ml^{-1}) and N represents the plate count after pressure treatment.

accelerate germination while others may protect germinated spores against inactivation. In the present work, the protective effect of milk constituents against thermal inactivation of DPA-free spores could predominate.

Whatever the suspension medium, spore inactivation increased as the three factors of pressure, temperature and pressure-holding time increased. The model showed significant factor interaction meaning that significant synergistic effects occur with pressure–temperature and pressure–pressure-holding time in distilled water and in milk. At temperatures higher than 40 °C, the treatment pressure and pressure-holding time may be decreased to obtain the same inactivation level. In the same way, for pressure levels higher than 400 MPa, the treatment temperature and the processing time may be decreased to provide the same inactivation level. Nevertheless, significant inactivation requires minimal values of pressure and temperature while the pressure-holding time has only a significant effect for pressure treatments above 400 MPa and above 40 °C. Thus, the treatment conditions must be severe enough both to induce the germination process and to inactivate the germinated spores.

Several authors have stated that a considerable (more than 5-log cycle) and reproducible reduction of spores by pressure of around 600 MPa is only possible in combination with temperatures between 75 and 80 °C (Cléry-Barraud et al., 2004; Gao et al., 2006; Margosch et al., 2004). For example, the optimum process parameters for a 6-log cycle reduction of *Geobacillus stearothermophilus* were calculated using RSM to be 625 MPa, 86 °C and 14 min (Gao et al., 2006). Moerman, Mertens, Demey, and Huyghebaert (2001) demonstrated a 5–6-log cycle reduction of *Geabacillus stearothermophilis* spores after processing at 400 MPa/80 °C for 60 min. According to Gao et al. (2006), a 6-log cycle reduction of *B. subtilis* spores in milk was obtained with a treatment at 576 MPa, 87 °C for 13 min. The present study shows that, for *B. sporothermodurans* LTIS27 spores, a 5-log cycle inactivation can be obtained with less drastic treatment conditions of 477 MPa/48 °C for 26 min and 495 MPa/49 °C for 30 min, in distilled water and in milk, respectively.

B. sporothermodurans spores are known to be highly heat-resistant with D_{140} values ranging from 3.4 to 7.9 s in skim milk (Huemer, Klijn, Vogelsang, & Langeveld, 1998). Using the data obtained by Moerman et al. (2001), we compared the inactivation of *B. sporothermodurans* in milk with the inactivation of other highly heat-resistant spores, the spores of *G. stearothermophilus* suspended in fatty meat. After the same treatment at 400 MPa, 50 °C for 30 min, 1.52 and 3.68-log cycle were obtained with the model developed by Moerman et al. (2001) on *G. stearothermophilus* inactivation and the model of the present study on *B. sporothermodurans*, respectively. Although the compared results concern only one specific strain of each bacteria, this could mean that the heat resistance of *B. sporothermodurans* spores has no relation to its pressure resistance. In the literature, two opinions coexist about the relationship between the pressure and heat

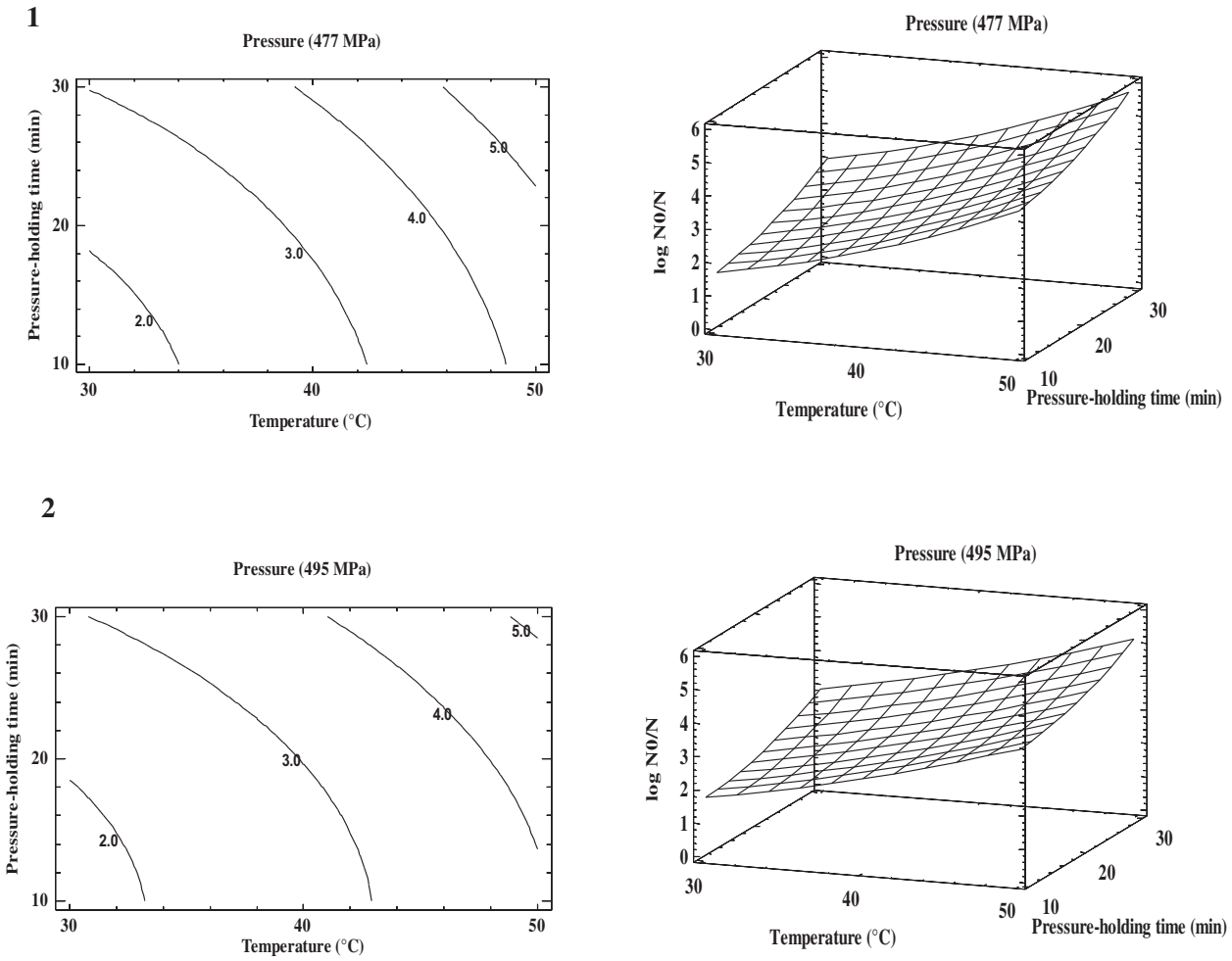

Fig. 3. Response surface and contour plots showing the effects of temperature and pressure-holding time on the inactivation of *B. sporothermodurans* LTIS27 spores. The data on the contour maps are log N_0/N in distilled water (1) and milk (2). N_0 represents the plate count of the untreated spore suspension (5×10^7 spore ml^{-1}) and N represents the plate count after pressure treatment.

resistance of spores. Rocken and Spicher (1993) demonstrated that the highest resistance to pressure was observed in highly heat-resistant strains of *B. subtilis* and *B. amyloliquefaciens* previously isolated from ropy bread. This finding may indicate a correlation between heat resistance and pressure resistance. However, spores of *B. amyloliquefaciens* are considerably more pressure-resistant than spores of *G. stearothermophilus*, which exhibit a higher resistance to wet heat (Ananta, Heinz, Schluter, & Knorr, 2001). Furthermore, Nakayama et al. (1996), by comparing the pressure and heat resistance of spores from six *Bacillus* strains, indicated that, for some strains, the pressure resistance did not correlate well with the heat resistance values obtained. Therefore, no pressure treatment condition could be converted into a heat sterilization condition. The sporulation medium, pressure-transmitting fluid, initial treatment temperature, and the high pressure equipment may all have a strong influence on the resistance of *Bacillus* spores to combined HP-heat treatments (Nakayama et al., 1996).

Clearly, the HP-resistance of *Bacillus* species is highly variable. Margosch et al. (2004), while studying the levels of resistance of 14 food isolates and 5 laboratory strains of *Bacillus* species to combined pressure and temperature treatments, found a large variation in the pressure resistance of spores. The reduction by treatment at 800 MPa and 70 °C for 4 min ranged from more than 6 log units for *B. subtilis* and *B. licheniformis* to no reduction for *B. amyloliquefaciens*. For vegetative cells, it has been reported that different microbial species, and even strains within species, can exhibit different HP sensitivity (Patterson, Quinn, Simpson, & Gilmour, 1995). This may also be true for bacterial spores. Furthermore, HP-induced spore inactivation depends on a range of factors, related to the microorganism itself, the conditions of treatment (levels of pressure, treatment time, and temperature) and to the medium in which the microorganisms are suspended. Recently, Minh, Durand, Loison, Perrier-Cornet, and Gervais (2011) also showed that the sporulation conditions may affect the HP-resistance of spores. Such variability makes the modeling of the HP-inactivation of spores a very difficult challenge.

5. Conclusion

RSM involving experimental design and regression analysis was used to reveal the influence of pressure, temperature, and pressure-holding time on the inactivation of *B. sporothermodurans* spores in distilled water and in skim milk. Our results suggest that the association of heat with pressure is very efficient for the inactivation of *B. sporothermodurans*, as has been shown for other sporeforming species, more so in distilled water than in milk. The optimum process parameters obtained for a 5-log cycle reduction of *B. sporothermodurans* spores were 477 MPa/48 °C for 26 min in distilled water and 495 MPa/49 °C for 30 min in milk. This study shows the potential of using HP in combination with moderate

temperature to inactivate *B. sporothermodurans* spores in milk. It is particularly interesting because the initial contamination density (N_0) of HRS spores in raw milk is generally lower than 10^5 cfu ml^{-1}. However, for scale-up process validation, a multiple-strain study should be performed. Finally, this work opens up new perspectives for the study of *B. sporothermodurans* spore behavior under HP.

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