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Heat Resistance of Spore-forming Microorganisms

(*Bacillus sporothermodurans, Bacillus subtilis and Geobacillus stearothermophilus*)

Under Isothermal and Non-isothermal Conditions.

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Cartagena, Septiembre, 2014

Heat resistance of spore-forming microorganisms (*Bacillus sporothermodurans* IIC65*, Bacillus subtilis* IC9*, Geobacillus stearothermophilus* T26) under isothermal and non-isothermal conditions.

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Que el trabajo titulado "**Heat resistance of spore-forming microorganisms (*Bacillus sporothermodurans* IIC65, *Bacillus subtilis* IC9, *Geobacillus stearothermophilus* T26) under isothermal and non-isothermal conditions**", ha sido realizado por Dª. Isabel Gómez Jódar, bajo la dirección y supervisión de D. Alfredo Palop Gómez y Dª. María Ros Chumillas y que se autoriza al alumno a la defensa del mismo.

En Cartagena, a 15 de septiembre de 2014

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INDEX

RESUMEN

El principal género de microorganismos esporulados altamente resistentes al calor involucrados en el deterioro de alimentos es *Bacillus*. Este género causa problemas de no esterilidad en alimentos enlatados y reduce la vida comercial de muchos alimentos procesados. En este estudio se determinó la termorresistencia de *Bacillus sporothermodurans* IIC65, *Bacillus subtilis* IC9 y *Geobacillus stearothermophilus* T26 mediante un termorresistómetro Mastia (Conesa *et al*., 2009). Las determinaciones de termorresistencia se realizaron bajo condiciones isotérmicas (a 120ºC, 122,5ºC, 125ºC y 127,5ºC), bajo condiciones no isotérmicas (a velocidades de calentamiento de 1ºC/min y 20ºC/min) y bajo condiciones más complejas (tratamiento con etapas de calentamiento, mantenimiento y enfriamiento a una velocidad de calentamiento/enfriamiento de 27,5ºC/min). Los resultados mostraron que *Geobacillus stearothermophilus* no fue tan resistente al calor como se pensaba; *Bacillus sporothermodurans* y *Bacillus subtilis* presentaron hombros en las curvas de supervivencia y fueron más termorresistentes que lo que se predijo mediante el modelo de Weibull, mientras que *Geobacillus stearothermophilus* presentó colas en las curvas de supervivencia y fue menos termorresistente que lo que se predijo mediante el modelo de Weibull; y la inactivación más rápida fue lograda a una velocidad de calentamiento de 20ºC/min. Además, la inactivación que se consiguió bajo el tratamiento térmico más complejo (calentamiento-mantenimiento-enfriamiento) fue baja comparada con la inactivación que se logró a 1ºC/min y 20ºC/min para *Bacillus sporothermodurans* y *Bacillus subtilis*.

Palabras clave: *Bacillus sporothermodurans*; *Bacillus subtilis*; *Geobacillus stearothermophilus*; Esporas; Termorresistencia; Cinética de inactivación

ABSTRACT

The main genus of heat-resistant spore-forming bacteria involved in food spoilage is *Bacillus*. Mostly, heat-resistant spore-forming *Bacillus* cause non-sterility problems in canned food and reduce the shelf life of many processed foods. In this study the heat resistance of *Bacillus sporothermodurans* IIC65*, Bacillus subtilis* IC9 *and Geobacillus stearothermophilus* T26 was determined by a thermoresistometer Mastia (Conesa *et al*., 2009) under isothermal conditions at 120ºC, 122.5ºC, 125ºC and 127.5ºC, non-isothermal conditions at heating rates of 1ºC/min and 20ºC/min and under more complex conditions (heating, holding and cooling periods) at a heating/cooling rate of 27.5ºC/min. The results showed that *Geobacillus stearothermophilus* was not as heat resistant as is thought; *Bacillus sporothermodurans* and *Bacillus subtilis* presented shoulders in their survival curves and they were always more heat resistant than predicted by Weibull model while *Geobacillus stearothermophilus* presented tail phenomena and it was always less heat resistant than predicted by Weibull model; and the fastest inactivation was achieved at a heating rate of 20ºC/min. In addition, the inactivation achieved under a complex thermal treatment (heating-holding-cooling) was low compared to that achieved at 1ºC/min and 20ºC/min for *Bacillus sporothermodurans* and *Bacillus subtilis*.

Keywords: *Bacillus sporothermodurans*; *Bacillus subtilis*; *Geobacillus stearothermophilus*; Spores; Heat resistance; Inactivation kinetics

1. INTRODUCTION

The genus *Bacillus*, frequently isolated from soil, water, plants and animals, has the capability to form spores. Bacterial spores are defined as cells in a dormant state when environmental conditions are not optimal for cell growth. *Bacillus* spores are not just resistant to damaging agents such as desiccation, radiation, acids and chemical disinfectants but also are resistant to heat, which could be related to the low water content that spores contain. In addition, some *Bacillus* species produce hydrolytic enzymes in order to decompose polysaccharides, nucleic acids and lipids, allowing the use of these products as carbon source by microorganisms (Prescott *et al*., 1999). Therefore, as they are able to resist the heat and produce hydrolytic enzymes, these kinds of microorganisms are considered food spoilage bacteria.

There are several treatments to restrict the growth of food spoilage bacteria, and hence to prolong food shelf-life, although heat treatments are still the most widely used method to preserve food shelf life. The spoilage of canned food because of the persistence of non-pathogenic and highly heat resistant spore-forming bacteria is still an industrial and economical risk (Logan and De Vos., 2009; Burgess *et al*., 2010; Prevost *et al*., 2010; Rigaux *et al*., 2014) so food companies need to be very careful about these heat resistant spore-forming bacteria when applying the heat treatment.

The main species of heat resistant spore-forming microorganisms involved in food spoilage belong to the genus *Bacillus*, such as *Bacillus sporothermodurans, Bacillus subtilis,* but there are also other heat resistant species, belonging to other closely related genera, such as *Geobacillus stearothermophilus*. *B. sporothermodurans* is a heat resistant mesophilic spore-forming bacterium causing non-sterility problems in canned food (Huemer *et al*., 1998; Oomes *et al*., 2007; van Zuijlen *et al*., 2010; Esteban *et al*., 2013). It is frequently found in UHT milk (Huemer *et al*., 1998). *B. subtilis* is a common cause of food spoilage and their heat resistant spores often pose a challenge to thermal efficacy of heat processes resulting in reduced shelf life of many processed foods (Jagannath *et al*., 2005). *G. stearothermophilus* is recognized as a major source of spoilage in canned food and, since it is a thermophilic

microorganism, it is frequently detected in cans presenting defects after a 7-day incubation at 55ºC (André *et al*., 2013; Rigaux *et al*., 2014). It is very useful as indicator microorganism for validation studies of thermal processes such as sterilization.

In recent years, occurrence of more heat resistant spores often evoked even more severe heat treatments (Oomes *et al*., 2007; van Zuijlen *et al*., 2010). Food companies tend to increase the treatment temperature, as well as the time, to try to kill these heat resistant microorganisms and their spores. However, increasing the treatment temperature affects the quality of food, since the higher the treatment temperature, the lower the sensory quality and, as a result, over-processed products are obtained. Nevertheless, increasing the processing temperature (linked with the corresponding reduction of the time required for the treatment) is less damaging for the components of food, and still maintains the lethal effect for microorganisms, leading to an improvement of the quality of food with the same level of sterility (Palop *et al*., 1999). This is the reason why it is very important to know the microbial inactivation kinetics, which allows obtaining the right combination of time and temperature required for the death of microorganisms and destruction of their spores, avoiding the over-processing of products.

There are several methods to determine the kinetics of the death rate of microorganisms. The traditional approach considers that microorganisms within a population are identical and their inactivation follows first-order kinetics. However, it seems likely that microbial inactivation may not always follow first-order kinetics as deviations from linearity of survival curves, such as shoulders or tails, have been reported by several authors. Some authors (Peleg and Cole, 1998; Fernandez *et al*., 1999) explain this behavior assuming that, at a given temperature, the time of exposure to heat, which causes the death of a microbial cell or a bacterial spore, is variable from one individual to the other, and that the dispersion of individual heat resistances is governed by a frequency distribution. The survival curves of highly heat-resistant spore-forming bacteria frequently present shoulders (Esteban et al., 2013). The presence of shoulders in survival curves can be successfully described using frequency distributions such as Weibull (Peleg and Cole, 1998). The

mathematical model based on the Weibull distribution is a simple and flexible model which only uses two parameters to describe both concave upward or downward survival curves as well as linear survival curves. On the contrary, the rest of alternative models to the first order approach are very complex because they use many parameters to describe survival curves of a unique shape. Furthermore, the Weibull model has been successfully used to model microbial inactivation by heat (van Boekel, 2002).

The effect of the heating and cooling rate has been scarcely studied. Still, some authors have used non-isothermal methods as an alternative to the study of microbial inactivation kinetics (Periago *et al*., 1998; Fernández *et al*., 1999; Conesa *et a*l., 2003; Peleg and Normand, 2004; Hassani *et al*., 2007; Esteban *et al*., 2013).

It seems that a fast heating rate can provide a lower heat resistance than expected in heat-resistant spore-forming microorganisms so the aim of this research was the study of the heat resistance of *B. sporothermodurans, B. subtilis* and *G. stearothermophilus* (heat-resistant spore-forming microorganisms frequently involved in food spoilage) under isothermal conditions in order to predict then the survival under non-isothermal conditions and complex treatments (simulating those applied in industry) and finally be able to compare the possible influence of the heating and cooling rate on the inactivation of heat-resistant spore-forming microorganisms.

2. MATERIALS AND METHODS

2.1 Bacteria strain and sample preparation

B. sporothermodurans IIC65, *B. subtilis* IC9 and *G. stearothermophilus* T26 (kindly supplied by Unilever Research Vlaardingen, The Netherlands) were used in this study. These microorganisms were isolated from canned soups produced by the company. Petri dishes of plate count agar (PCA; Scharlau, Barcelona, Spain) were inoculated with the microorganisms and incubated at 30ºC, 37ºC and 55ºC during 24 h for *B. sporothermodurans*, *B. subtilis* and *G. stearothermophilus* respectively. For each microorganism, four pure colonies

were taken from the agar plate and suspended in physiological salt solution (NaCl; Panreac, Barcelona, Spain) at 0.85%. Then, agar plates containing Campden Sporulation Agar (CSA; Brown *et al*., 1984) were surface inoculated with 0.2 mL of this suspension and incubated at 37ºC for *B. sporothermodurans* and *B. subtilis* and at 55ºC for *G. stearotermophilus.* After 5-7 days of incubation more than 90% of sporulation rate was achieved, as determined by a phase contrast microscopy (Leica, Wetzlar, Germany). Then the spores were collected by flooding with sterile distilled water and scratching the surface with a sterile spatula. After collecting, the spore suspensions were washed three times by centrifugation at 3000xg for 20 min. The concentration of spores in the final suspension was adjusted to 10^9 spores mL^{-1} with sterile distilled water. The suspensions were stored at 4ºC for at least two weeks before being used to establish their heat resistance to allow spore maturation (van Zuijlen *et al*., 2010).

2.2 Heat resistance determinations

Heat resistance determinations were carried out in a thermoresistometer Mastia (Conesa *et al*., 2009).

The main vessel (400 mL) was filled up with the heating medium which, in this study, was distilled water. Firstly, the heating medium was sterilized by heating at 135ºC in the thermoresistometer and after a few minutes the treatment temperature was programmed. Once the treatment temperature had attained stability the heating medium was inoculated with 0.2 mL of the spore suspension by a Hamilton-type syringe. Then, samples for each treatment time were collected into sterile tubes at preset intervals. Each sample was immediately cooled down with ice and was properly diluted in distilled water (dilution medium). Finally, dilutions were plated in Tryptic Soy Agar (TSA, Scharlau) and were incubated for 24 h at 37ºC (*B. sporothermodurans* and *B. subtilis)* or at 55ºC (*G. stearothermophilus)*.

The temperatures of the isothermal treatments for each microorganism were: 120, 122.5, 125 and 127.5ºC. For the non-isothermal treatments the thermoresistometer was programmed to start at an initial temperature of 90ºC which was increasing according to preset heating rates (1ºC/min and 20ºC/min).

Furthermore, more complex heat treatments (heating, holding and cooling periods) were also performed for each microorganism. For *B. sporothermodurans* and *B. subtilis* the thermoresistometer was programmed to start at an initial temperature of 82.5ºC, which was increased to 125ºC at a heating rate of 27.5ºC/min. This temperature was held for 1.5 min, and finally it was cooled down to 95ºC at the same cooling rate of 27.5ºC/min. For *G. stearothermophilus* the thermoresistometer was programmed to start at an initial temperature of 82.5ºC, which was increased to 122.5ºC. This temperature was held for 1.5 min, and finally it was cooled down to 92.5ºC, being the heating/cooling rates also of 27.5ºC/min.

Each resistance determination was carried out at least twice in independent experiments on different days.

2.3 Data analysis

2.3.1 Bigelow model

Bigelow model is the traditional approach describing inactivation kinetics. It is based on the statement that microbial inactivation follows first-order kinetics. The model results in the following equation (van Boekel, 2002):

$$\ln N = \ln N_0 - kt; \quad (1)$$

with N as the number of microorganisms, N_o as the initial number of microorganisms, k as the first-order rate constant (units in min^{-1} or s^{-1}) and t as the exposure time. This equation is then rearranged into:

$$\log \frac{N}{N_0} = \log S(t) = -\frac{t}{D}; \quad (2)$$

where D is the treatment time needed for one logarithmic reduction in the number of survivors (D = 2.303 / k ; units in min or s) and $S(t)$ is the survival ratio (N/N_0). For each experiment the logarithms of the survival ratio (log $S(t)$) were plotted vs the exposure time (t) to heat. D value is the inverse of the slope of the regression line of each graph. At a certain temperature, the higher the D value, the higher the thermal resistance of the microorganism.

The temperature dependence of *D* is usually expressed in the so-called *z* value (van Boekel, 2002). *z* value is the increase of temperature (ºC) to obtain one logarithmic decrease in the *D* value. For each microorganism the logarithm of the *D* values at different temperatures (log D_T) were plotted vs the different treatment temperatures (*T*) in the corresponding thermal death time curves. *z* value is the inverse of the slope of the regression line of that graph.

Whether deviations from linearity of first-order kinetics, such as shoulders or tails, are found in the survival curves, only the data points which are in the straight region of the curves are taken into account in order to obtain the regression line. The graphs from which the *D* values were obtained had at least four points in the straight region of the curve and a coefficient of determination above 0.85.

2.3.2 Weibull model

The Weibull model has been successfully used to describe the nonlinear inactivation of different microorganisms. In this study the cumulative form of the Weibull distribution (Mafart et al, 2002) was used:

$$\log N = \log N_0 - \left(\frac{t}{\delta}\right)^p; \quad (3)$$

where *N* is the number of spores after the heat treatment (cfu/mL), N_0 the number of spores at initial time (cfu/mL), *t* the treatment time (min) and δ and *p* are two characteristic parameters of the Weibull distribution: the δ value is the time of the first decimal reduction and the *p* value is the shape parameter which depends on the shape of the survival curve: $p < 1$ for concave upwards survival curves, $p = 1$ for linear survival curves and $p > 1$ for concave downwards survival curves.

In this study, we used a rate model derived from Eq. (3), representing the momentary time-dependent isothermal logarithmic inactivation rate, which can be written as an ordinary differential equation as given by Eq. (4) (van Zuijlen *et* al., 2010):

$$\frac{dN}{dt} = -p \cdot \left(\frac{t}{\delta}\right)^p \cdot t^{p-1}; \quad (4)$$

with the initial condition: $N(_0) = N_0$.

A single *p* value for all survival curves obtained for the same microorganism was used as proposed by Couvert *et* al. (2005). For the global optimisation, Eq. (5) was incorporated into Eq. (3) and the parameter *p* was fitted in common, whereas the initial states were fitted for each curve.

The prediction of survivors under non isothermal experiments was based on the dependence of δ with respect to temperature, which can be described with the classic Bigelow model as given by Eq. (5) (Mafart *et* al., 2002):

$$\delta(T) = \delta_{T_{ref}} \cdot 10^{-\frac{T-T_{ref}}{z}}; \quad (5)$$

Where $\delta_{T_{ref}}$ is the δ (*T*) value at the reference temperature (T_{ref}), and *z* is the number of degrees Celsius change of temperature required to achieve a tenfold change in δ value. The reference temperature used for the survival prediction under non-isothermal and more complex conditions was 125ºC.

2.4 Statistical analysis

The linear regressions, the coefficients of determination (R^2), the mean of the data, the standard deviation (SD), the root mean square errors of predictions (RMSE) and the 95% confidence intervals (CI) were calculated using Windows Microsoft® Excel 2007.

The non-linear regressions and the parameters (δ and *p*) of Weibull model were adjusted using the Solver tool of Excel.

3. RESULTS AND DISCUSSION

3.1 Heat resistance under isothermal conditions

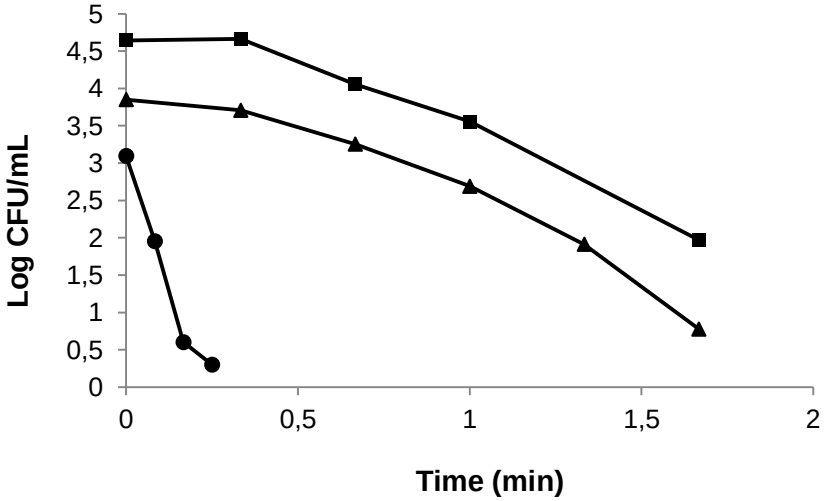

Fig.1. Survival curves of *B. sporothermodurans* IIC65 ( ), *B. subtilis* IC9 ( ) and *G. stearothermophilus* T26 ( ) obtained at 127.5ºC.

Table 1. *D* values (min) of *B. sporothermodurans* IIC65, *B. subtilis* IC9 and *G. stearothermophilus* T26 obtained under isothermal conditions.

Microorganism / T (ºC)	*D* value (min)	RMSE	R^2	Mean *D* value ± SD
Bacillus sporothermodurans IIC65				
120ºC	3.64	0.31	0.94	3.69 ± 0.07
120ºC	3.75	0.08	0.98	
122,5ºC	2.30	0.27	0.97	2.25 ± 0.06
122,5ºC	2.21	0.06	0.99	
125ºC	1.01	0.37	0.96	1.04 ± 0.04
125ºC	1.07	0.06	0.99	
127,5ºC	0.49	0.14	0.99	0.48 ± 0.01
127,5ºC	0.47	0.18	0.98	
Bacillus subtilis IC9				
120ºC	5.24	0.12	0.98	5.24
122.5ºC	1.64	0.22	0.96	1.79 ± 0.21
122.5ºC	1.94	0.12	0.98	
125ºC	1.18	0.24	0.92	1.10 ± 0.11
125ºC	1.02	0.16	0.98	
127.5ºC	0.46	0.24	0.96	0.44 ± 0.02

127.5ºC	0.43	0.16	0.98	
Geobacillus stearothermophilus T26				
120ºC	2.71	0.27	0.85	2.69 ± 0.02
120ºC	2.67	0.23	0.90	
122.5ºC	0.74	0.20	0.95	0.74
125ºC	0.41	0.05	0.99	0.33 ± 0.11
125ºC	0.25	0.16	0.98	
127.5ºC	0.13	0.17	0.97	0.11 ± 0.03
127.5ºC	0.08	0.08	0.99	

The data obtained showed that *B. sporothermodurans* and *B. subtilis* were the most heat-resistant spore-forming microorganisms of those studied while *G. stearothermophilus* was the less heat resistant of those studied as it presented a mean $D_{127.5}$ value of 0.11 min (Table 1) which is much lower than the mean $D_{127.5}$ value of *B. sporothermordurans* (0.48 min; Table 1) and the mean $D_{127.5}$ value of *B. subtilis* (0.44 min; Table 1). However it is usually thought that the spores of *G. stearothermophilus* are extremely resistant to high temperatures as it is frequently detected in cans showing defects after the standard incubation test, a 7-day incubation at 55ºC (André *et al*., 2013). As a consequence, it is the most frequently applied organism to select conditions for thermal sterilisation of food (Lynch and Potter, 1988; Rodrigo *et al*., 1999; Iciek *et al*., 2000; Tejedor *et al*., 2001). It has been shown that *B. sporothermodurans* has a considerably high heat resistance at UHT temperatures with D_{140} value from 3.4-7.9 s, compared with *G. stearothermophilus* with a D_{140} value of 0.95 s (Huemer *et al*., 1998). It has also been reported that the heat resistance of *B. sporothermodurans* and *B. subtilis* is very similar (Huemer *et al.*, 1998). Our results agree with those, since no significant differences were found between the *D* values for both microorganisms (Table 1). Moreover, it can be found in the literature that, because of their high heat resistance, the spores of *B. sporothermodurans* would be more suitable for process validation than the spores of *G. stearothermophilus* (van Zuijlen *et al*., 2010).

As the survival curves both of *B. sporothermodurans* and *B. subtilis* showed shoulders (Fig. 1) it means that the inactivation kinetics of these

microorganisms does not follow first-order kinetics. The presence of shoulders in survival curves of highly heat-resistant spore-forming bacteria has been frequently described in the literature (Palop *et al*., 1999; Jagannath *et al*., 2005; van Zuijlen *et al.*, 2010; Esteban *et al.*, 2013). These shoulders are the reason why only the linear portion of the survival curves was taken into account to fit the data by the Bigelow linear model. Still, since the survival data of these microorganisms do not follow first-order kinetics, these data were also fitted by the Weibull non-linear model (Eq. (3)), which takes into account the shape of the whole survival curve. The survival curves of *B. sporothermodurans* and *B. subtilis* showed shoulders (*p* values of 1.46 and 1.85 respectively; Table 2; Fig. 1), while the survival curves of *G. stearothermophilus* were concave upwards (*p* value = 0.82; Table 2; Fig. 1) showing tail phenomena.

Table 2. δ (min) and *p* values of *B. sporothermodurans* IIC65, *B. subtilis* IC9 and *G. stearothermophilus* T26 obtained under isothermal conditions.

Microorganism / T (ºC)	δ value (min)	*p* value	RMSE	R^2	Mean *D* value $\pm$ SD
Bacillus sporothermodurans IIC65					
120ºC	6.17		0.51	0.92	8.02 $\pm$ 2.62
120ºC	9.87		0.32	0.91	
122,5ºC	4.56		0.45	0.91	4.95 $\pm$ 0.55
122,5ºC	5.34	1.46	0.62	0.84	
125ºC	1.41		1.44	0.96	1.82 $\pm$ 0.58
125ºC	2.24		0.67	0.88	
127,5ºC	0.87		0.32	0.93	0.78 $\pm$ 0.12
127,5ºC	0.69		0.29	0.98	
Bacillus subtilis IC9					
120ºC	10.93		0.48	0.82	10.93
122.5ºC	4.49		0.30	0.92	4.32 $\pm$ 0.24
122.5ºC	4.15	1.85	0.23	0.98	
125ºC	2.18		0.42	0.93	2.15 $\pm$ 0.05

125ºC	2.11		0.15	0.94	
127.5ºC	0.91		0.06	0.93	0.97 ± 0.08
127.5ºC	1.03		0.37	0.88	
Geobacillus stearothermophilus T26					
120ºC	2.18		0.38	0.89	2.14 ± 0.05
120ºC	2.10		0.36	0.91	
122.5ºC	0.58	0.82	0.15	0.99	0.58
125ºC	0.36		0.19	0.99	0.28 ± 0.11
125ºC	0.20		0.22	0.98	
127.5ºC	0.19		0.80	0.97	0.12 ± 0.08
127.5ºC	0.06		0.40	0.95	

It is common to find in literature that survival curves of *Bacillus* species have *p* values above 1 (Mafart *et al*., 2002) such as those obtained for *B. sporothermodurans* and *B. subtilis* in this study. Still, *p* values below 1 can also be found for *B. sporothermodurans* (Periago *et al*., 2004). Perhaps, the lack of heat activation prior to the heat resistance determinations in this research could explain, at least in part, the shoulder phenomena. On the other hand, *G. stearothermophilus* showed a *p* value lower than 1. These results agree with those obtained in other studies in which *G. stearothermophilus* showed concave upwards survival curves showing tailing phenomena (Iciek *et a*l., 2006; Iciek *et a*l., 2008).

In addition, it can also be observed that the δ value of *G. stearothermophilus* is lower than the δ value of *B. sporothermodurans* and *B. subtilis* (Table 2) as mentioned above with the data obtained by Bigelow model.

Regarding the *z* values obtained by Bigelow and Weibull model, *B. sporothermodurans* and *B. subtilis* presented very similar *z* values and they were much greater than the *z* value of *G. stearothermophilus* (Table 3; Fig. 2). As mentioned above some studies have already reported similarities in the heat resistance of *B. sporothermodurans* and *B. subtilis* (Huemer *et al*., 1998). On the other hand, the *z* values obtained for all the microorganisms

were different depending on the model used (Table 3) but no significant differences were found.

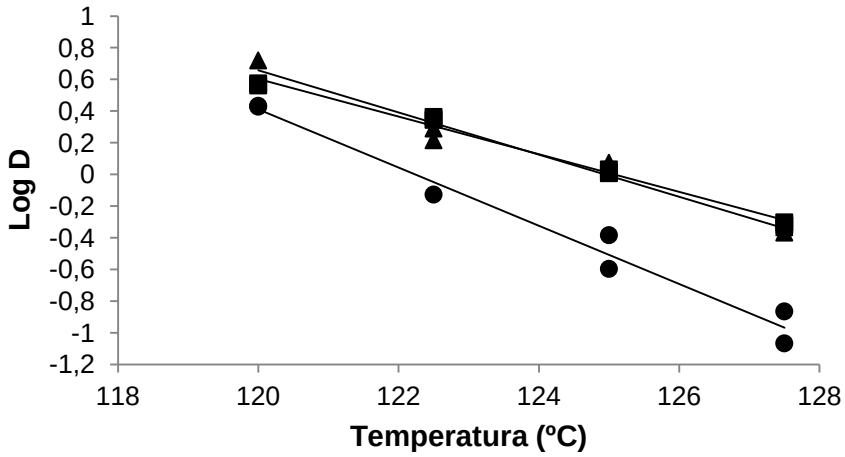

Fig.2. Log D vs Temperature (ºC) of *B. sporothermodurans* IIC65 (■), *B. subtilis* IC9 (▲) and *G. stearothermophilus* T29 (●).

Table 3. *z* values (ºC) of *B. sporothermodurans* IIC65, *B. subtilis* IC9 and *G. stearothermophilus* T26 obtained by Bigelow and Weibull model.

Microorganism	z	95%CI -	95%CI +	R^2
B. sporothermodurans IIC65				
Bigelow	8.40	7.62	9.34	0.99
Weibull	7.26	5.82	9.63	0.94
B. subtilis IC9				
Bigelow	7.50	6.26	9.37	0.97
Weibull	7.34	6.75	8.05	0.99
G. stearothermophilus T26				
Bigelow	5.44	4.61	6.64	0.97
Weibull	5.90	4.40	8.95	0.92

3.2 Heat resistance under non-isothermal conditions

The survival curves of *B. sporothermodurans* and *B. subtilis* obtained after the non-isothermal treatments at a heating rate of 1ºC/min (Figs. 3 and 5) showed a long shoulder indicating a long activation period. These

shoulders even showed an increase in the survivor counts, which may be indicative of the activation phenomenon taking place. However, under non-isothermal conditions at a similar heating rate of 1ºC/min, the survival curves of *G. stearothermophilus* (Fig. 7) showed a shorter shoulder without any increase in the survivor counts. About 30 minutes were needed to completely inactivate *G. stearothermophilus* compared to the 35-40 minutes required to inactivate *B. sporothermodurans* and *B. subtilis*. It seems like when shorter the time which these microorganisms need to get activated, shorter the time needed to kill them.

On the other hand, non-isothermal treatments at a heating rate of 20ºC/min (90-130ºC) were also performed for all the microorganisms (Figs. 4, 6 and 8). The survival curves of *B. sporothermodurans* and *B. subtilis* were similar to those obtained at a heating rate of 1ºC/min. The shoulders obtained could be regarded as “activation shoulders” (Corradini *et al*., 2010). The survival curve which shows an “activation shoulder” initially rises and then, after reaching a peak, it starts its decrease. This can be observed in the survival curves of *B. sporothermodurans* and *B. subtilis* obtained at both heating rates (1 and 20ºC/min; Figs. 3, 4, 5 and 6). That rise has been attributed to ‘dormant spores’ activation by their exposure to the high temperature. This explains why, after a short heat treatment even at an otherwise lethal temperature, the number of spores appears to have grown (Peleg, 2006). As the heating rate is fast, the microorganisms remain exposed to different temperatures very little time but temperatures increase so fast that allows for quick spore activation. The survival curves of all microorganisms obtained at a rate of 20ºC/min showed the same shape as those of the same microorganism obtained at a rate of 1ºC/min. The difference is that the inactivation was faster at a rate of 20ºC/min than that obtained at a rate of 1ºC/min. The time required of heat treatment at a rate of 20ºC/min to inactivate the microorganisms was about 2 minutes for *G. stearothermophilus* and about 3 minutes for *B. sporothermodurans* and *B. subtilis* showing again that *G. stearothermophilus* is the least heat resistant of the microorganisms in this study.

The heat resistance values obtained by Weibull model under isothermal conditions were used to predict the survival in the non-isothermal experiments at 1 and 20ºC/min. The results showed that the survival of *B. sporothermodurans* and *B. subtilis* was greater than predicted by Weibull both at the rate of 1ºC/min as at 20ºC/min (Figs. 3, 4, 5 and 6). However, the opposite happened with *G. stearothermophilus*, as its survival was lower than predicted by Weibull both at the rate of 1ºC/min as at 20ºC/min (Figs. 7 and 8).

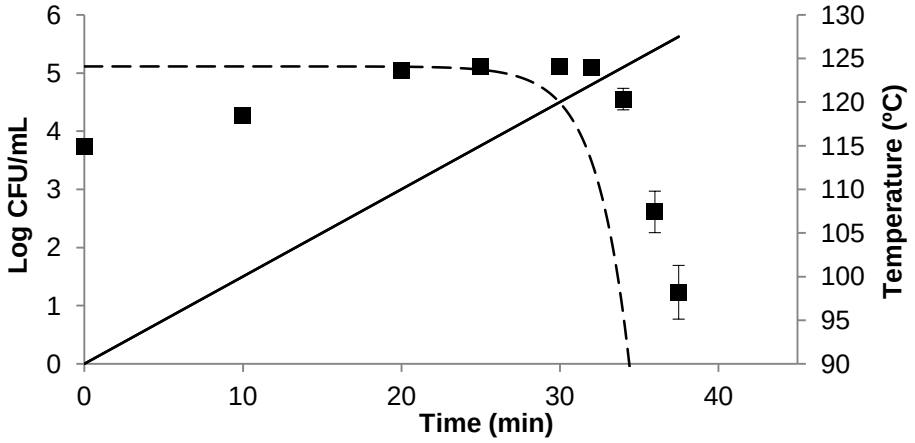

Fig.3. Survival curve of *B. sporothermodurans* IIC65 under non-isothermal conditions (90-127.5ºC) at a heating rate of 1ºC/min fitted by Weibull model (dashed line) and the profile of temperatures (continuous lines).

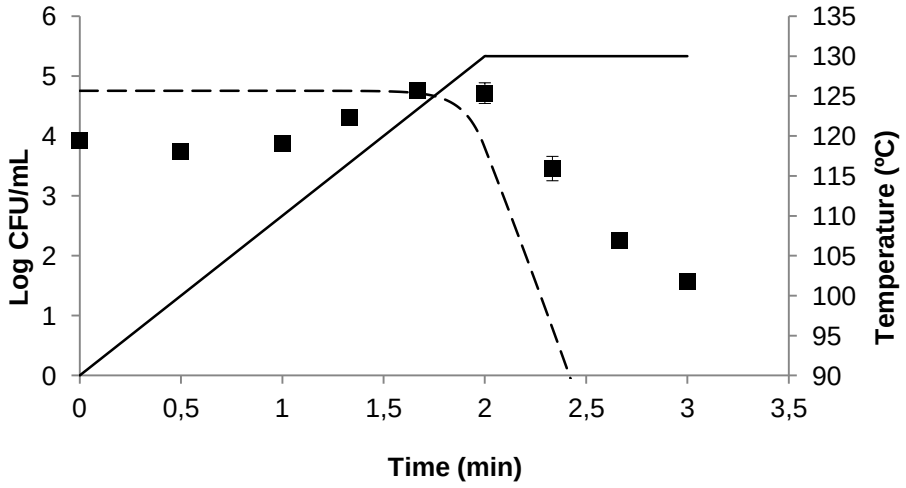

Fig.4. Survival curve of *B. sporothermodurans* IIC65 under non-isothermal conditions (90-130ºC) at a heating rate of 20ºC/min fitted by Weibull model (dashed line) and the profile of temperatures (continuous line).

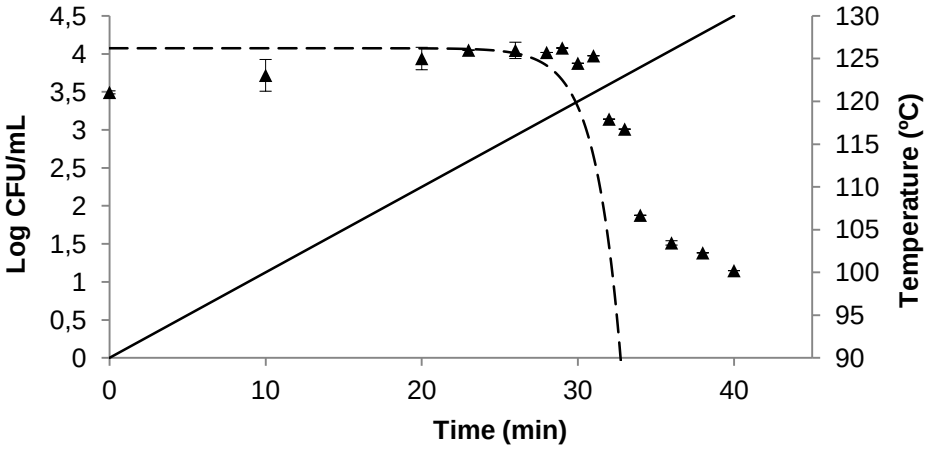

Fig.5. Survival curve of *B. subtilis* IC9 under non-isothermal conditions (90-130ºC) at a heating rate of 1ºC/min fitted by Weibull model (dashed line) and the profile of temperatures (continuous line).

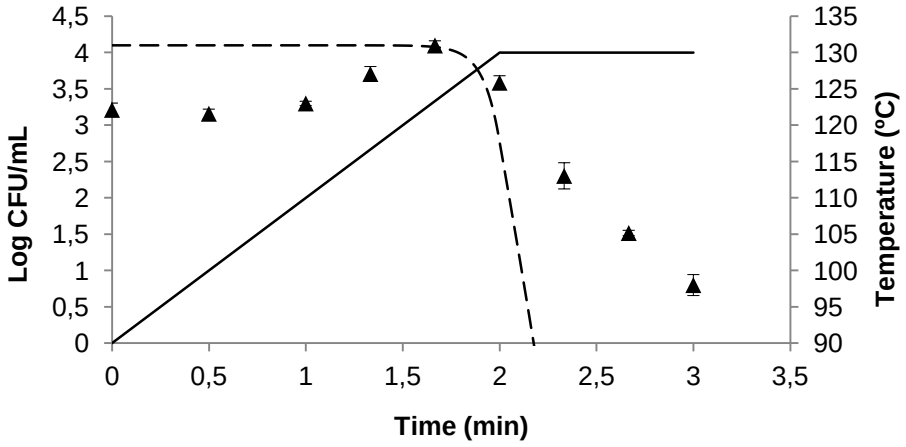

Fig.6. Survival curve of *B. subtilis* IC9 under non-isothermal conditions (90-130ºC) at a heating rate of 20ºC/min fitted by Weibull model (dashed line) and the profile of temperatures (continuous line).

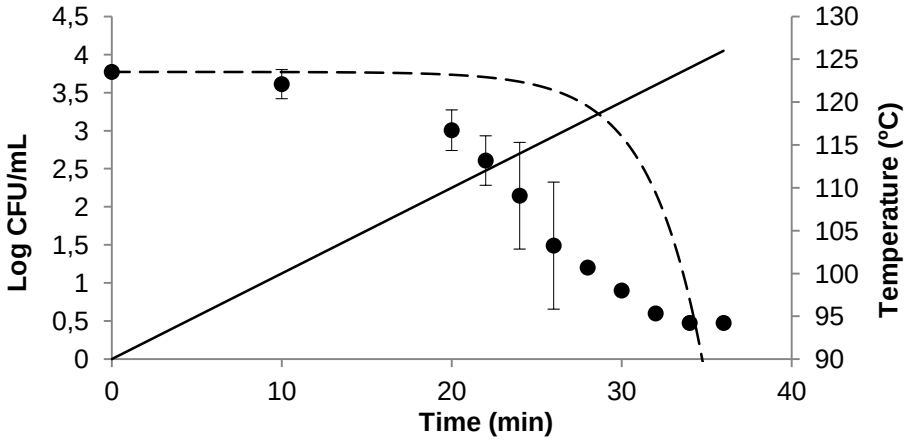

Fig.7. Survival curve of *G. stearothermophilus* T26 under non-insothermal conditions (90-126ºC) at a heating rate of 1ºC/min fitted by Weibull model (dashed line) and the profile of temperature (continuous line).

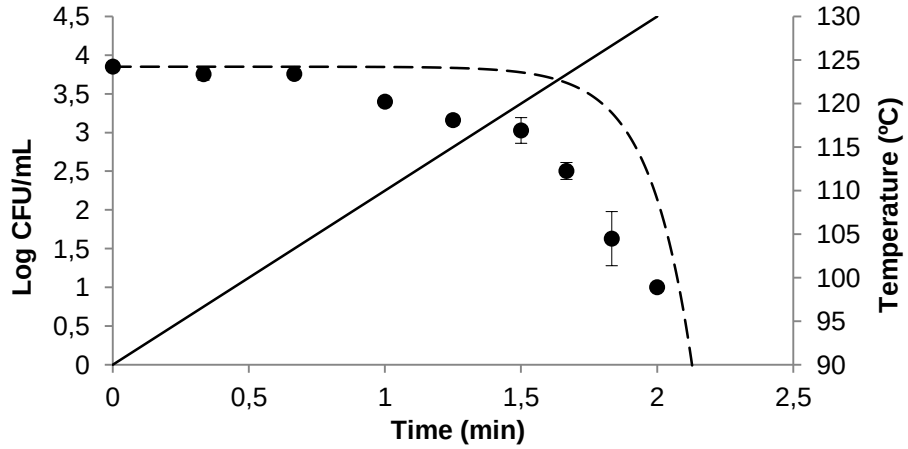

Fig.8. Survival curve of *G. stearothermophilus* T26 under non-isothermal conditions (90-127.5ºC) at a heating rate of 20ºC /min fitted by Weibull model (dashed line) and the profile of temperatures (continuous line).

Furthermore, more complex heat treatments (heating, holding and cooling periods) under non-isothermal conditions were performed for all the microorganisms in order to simulate thermal treatments that could be applied in the food industry. The heat resistance values obtained by Weibull model under isothermal conditions were used to predict the survival under these non-isothermal experiments at a heating rate of 27.5ºC/min. The survival curves of *B. sporothermodurans* and *B. subtilis* showed the same "activation shoulders" mentioned above (Figs. 9 and 10) than those obtained in the non-isothermal treatments both at a heating rate of 1ºC/min

as at 20ºC/min (Figs. 3, 4, 5 and 6). However in these complex heat treatments the inactivation achieved was much lower, since they were programmed to get some spore survival at the end of the treatment (see the predicted inactivation curves in the corresponding figures). The lower lethality was achieved with a lower holding temperature. This time predictions from the Weibull model fitted quite well the data obtained from *B. sporothermodurans* in contrast to the data obtained from *B. subtilis* which showed more survival than predicted as happened in the previous heat treatments. But the holding and cooling periods did not affect in the same way to the three microorganisms studied because in the case of *G. stearothermophilus* the survival curve obtained (Fig. 11) showed roughly the same shape as the survival curves obtained from the other heat treatments (Figs. 7 and 8) although the curve was more pronounced in this case. In addition, the inactivation achieved was the same or even greater than that obtained under the non-isothermal treatments, both at a heating rate of 1ºC/min as at 20ºC/min, and much greater than what was predicted by Weibull. It seems like microorganisms which require an activation time, the longer the time they need, the more heat resistant than predicted they are (such as *B. sporothermodurans* and *B. subtilis*). On the opposite, microorganisms with a short or non existing activation shoulder show lower heat resistance than predicted (such as *G. stearothermophilus*).

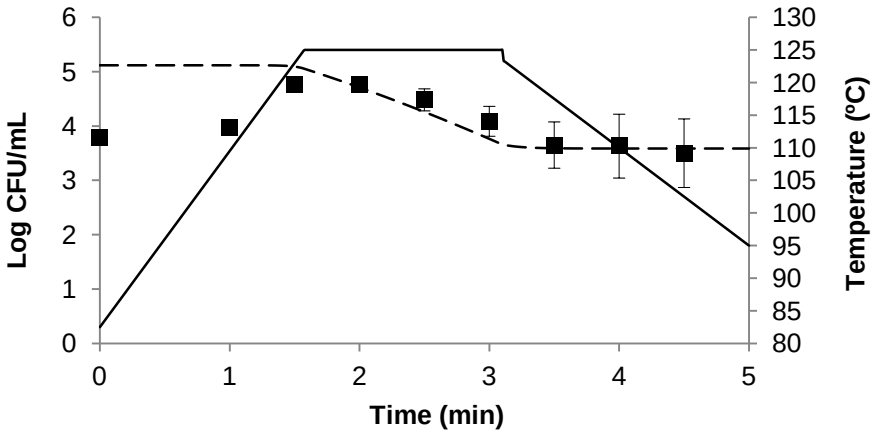

Fig.9. Survival curve of *B. sporothermodurans* IIC65 under non-isothermal conditions at a heating rate of 27.5ºC (heating from 82.5ºC until 125ºC, holding at

125ºC during 1.5 minutes and then cooling from 125ºC until 95ºC) fitted by Weibull model (dashed line) and the profile of temperatures (continuous line).

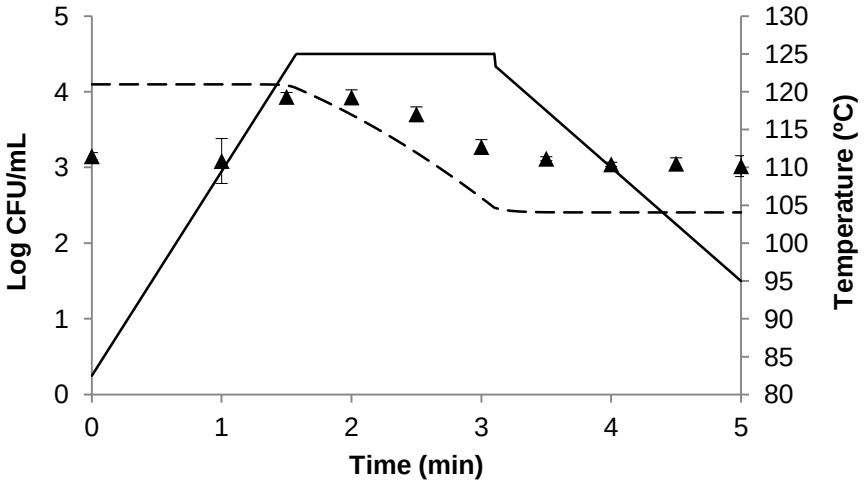

Fig.10. Survival curve of *B. subtilis* IC9 under non-isothermal conditions at a heating rate of 27.5ºC (heating from 82.5ºC until 125ºC, holding at 125ºC during 1.5 minutes and then cooling from 125ºC until 95ºC) fitted by Weibull model (dashed line) and the profile of temperatures (continuous line).

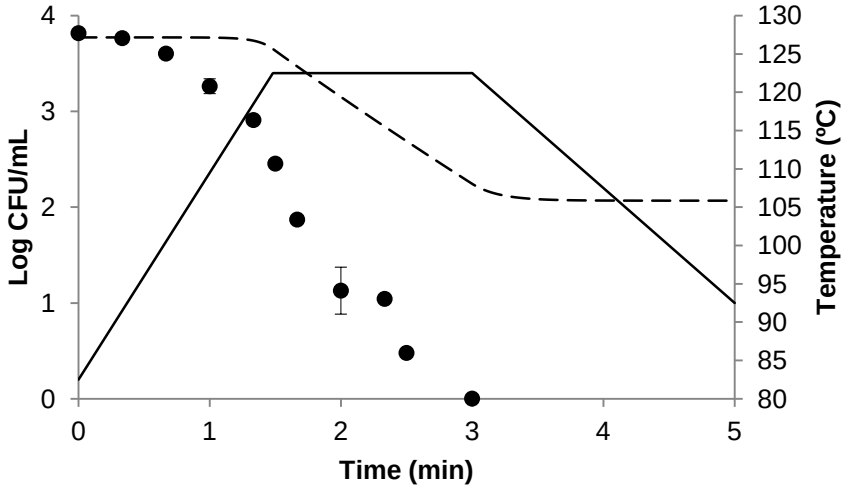

Fig.11. Survival curve of *G. stearothermophilus* T26 under non-isothermal conditions at a heating rate of 27.5ºC (heating from 82.5ºC until 122.5ºC, holding at 122.5ºC during 1.5 minutes and then cooling from 122.5ºC until 92.5ºC) fitted by Weibull model (dashed line) and the profile of temperatures (continuous line).

There is little evidence in the literature about the effect of the heating and cooling rate on highly heat-resistant spore-forming bacteria. Some studies have focused on the extraction of kinetic parameters from non-isothermal

data (Periago *et al*., 2004; Chen *et al*., 2007; Valdramidis *et al*., 2008) and others deal with modeling the inactivation under non-isothermal treatments (Peleg *et al*., 2001; Corradini *et al*., 2007) but there is little information about the behaviour of these microorganisms under non-isothermal conditions and the shoulders and survival curves obtained under such conditions. Yet, a recent study about the heat resistance of *B. sporothermodurans* IC4 under non-isothermal conditions at rates of 1ºC/min and 10ºC/min (Esteban *et al*., 2013) showed survival curves with shoulders which are not very similar to those obtained under non-isothermal conditions in this study. Despite this, the inactivation was similar in both studies, but 10-15 minutes were only required for the inactivation of *B. sporothermodurans* IC4 at 1ºC/min compared to the 40 minutes required in this study for the inactivation of *B. sporothermodurans* IIC65 at 1ºC/min. These differences could be because of the different initial temperature used, as these authors used 110ºC while in this study the initial temperature was set at 90ºC. Moreover, these differences could also be due to the different heating medium used in both studies, as it is known that the heating medium and its pH has a significant influence on the heat resistance of microorganisms (Fernández *et al*., 1996; López *et al*., 1996; Palop *et al*., 1999; Esteban *et al*., 2013). Regarding the fit by Weibull model these authors obtained a quite accurate fit of the experimental data with the prediction while in this study the experimental data were always above or under the prediction. The reason why Weibull did not fit the data well enough could be that it takes into account the length of the shoulder but not the spore activation. So far there are no models which take into account the spore activation time. Nevertheless, a review of existing predictive models and development of new models which allow predicting the microbial inactivation considering the time required for the spore activation would be quite necessary.

There are no data in the literature about heat resistance determinations of highly heat-resistant spore-forming bacteria under more complex heat treatments (heating, holding and cooling periods) simulating the conditions in the food industry to which the results obtained in this study can be compared. Further studies about the heat resistance of highly heat-resistant

spore-forming bacteria such as *B. sporothermodurans*, *B. subtilis* and *G. stearothermophilus* would be required in order to better understand the behaviour of these microorganisms under non-isothermal conditions, the effect of the spore activation time on the heat resistance and get a better interpretation of the survival curves and shoulders presented in such conditions.

4. CONCLUSIONS

In this study it was shown that *B. sporothermodurans* and *B. subtilis* were more heat resistant than *G. stearothermophilus* agreeing with some current studies which prove that the heat resistance of *G. stearothermophilus* is not as high as it was thought.

None of the microorganisms of this study followed first-order kinetics. Activation shoulders were shown in the survival curves of *B. sporothermodurans* and *B. subtilis,* which mean that these spore-forming microorganisms need time to get activated during the beginning of the heat treatment. On the other hand, tail phenomena were quite frequent in the survival curves of *G. stearothermophilus*.

Predictions for survivors under non-isothermal treatments using the Weibull model did not fit the data well enough. So the development of new predictive models which take into account the spore activation time would be a big step in the study of the heat resistance of highly heat-resistant spore-forming bacteria.

There is no a clear effect of the heating/cooling rate on the inactivation of spore forming microorganisms. But it could be observed that when microorganisms show activation shoulders under isothermal conditions, then the predictions are below of the count of survivors obtained under non isothermal conditions, while microorganisms which show tails under isothermal conditions, then the predictions are above of the count of survivors obtained under non isothermal conditions. Further studies about the heat resistance of highly heat-resistant spore-forming bacteria would be required in order to better

understand the behaviour of these microorganisms under non-isothermal conditions.

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