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Two distinct groups within the *Bacillus subtilis* group display significantly different spore heat resistance properties

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ABSTRACT

The survival of bacterial spores after heat treatment and the subsequent germination and outgrowth in a food product can lead to spoilage of the food product and economical losses. Prediction of time-temperature conditions that lead to sufficient inactivation requires access to detailed spore thermal inactivation kinetics of relevant model strains. In this study, the thermal inactivation kinetics of spores of fourteen strains belonging to the *Bacillus subtilis* group were determined in detail, using both batch heating in capillary tubes and continuous flow heating in a micro heater. The inactivation data were fitted using a log linear model. Based on the spore heat resistance data, two distinct groups ($p < 0.001$) within the *B. subtilis* group could be identified. One group of strains had spores with an average $D_{120\,^{\circ}C}$ of 0.33 s, while the spores of the other group displayed significantly higher heat resistances, with an average $D_{120\,^{\circ}C}$ of 45.7 s. When comparing spore inactivation data obtained using batch- and continuous flow heating, the *z*-values were significantly different, hence extrapolation from one system to the other was not justified. This study clearly shows that heat resistances of spores from different strains in the *B. subtilis* group can vary greatly. Strains can be separated into two groups, to which different spore heat inactivation kinetics apply.

1. Introduction

In the food industry, mesophilic, aerobic spore-forming bacteria are ubiquitously present (Anonymous, 2005; Gould, 2006). Their dormant endospores are highly resistant to environmental insults, and are able to survive various preservation regimes commonly used in the food industry. Heat treatment is commonly applied in food processing to inactivate bacteria and their spores. Insufficient heat treatment of bacterial spores may allow for survival of spores, potentially leading to food spoilage upon germination and outgrowth, and, in the case of food borne pathogens, to food poisoning (De Jonghe et al., 2010; Scheldeman et al., 2005). Depending on the spore heat resistance, heating regimes may exceed the required heat load, often negatively affecting product quality. Hence, knowledge on required heat load to inactivate spores in relation to product characteristics is important.

The heat resistance and germination properties of bacterial spores and their phenotypic variation are a major concern of the food industry (Eijlander et al., 2011; Hornstra et al., 2009). Different *Bacillus* species including *Bacillus cereus*, *Bacillus coagulans*, *Bacillus subtilis*, and *Bacillus sporothermodurans* are able to form highly heat resistant spores that can survive the heating regimes that are commonly used in food preservation (Scheldeman et al., 2006). Various spores belonging to the genera of *Bacillus*, *Aneurinibacillus*, and *Paenibacillus* are able to survive heat treatments of temperatures higher than 120 °C (te Giffel et al., 2002).

The heat resistance of spores can vary between species and even between strains of one species. Variation in spore heat resistance between different strains of *Bacillus sp.* has been reported, but not extensively studied. van Asselt and Zwietering (2006) indicated that strain variation in *B. cereus* significantly influences spore heat resistance. Another example is *B. sporothermodurans*, which produces spores that are highly heat resistant and can survive UHT treatments (Esteban et al., 2013; Huemer et al., 1998; van Zuijlen

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et al., 2010). For this bacterium, clear differences were observed in decimal reduction times (*D*-value) at 100 °C for spores of strains of various isolation sources (Scheldeman et al., 2006). Variation in spore heat resistances of strains of *B. subtilis* isolated from different soups has also been reported (Oomes et al., 2007). Kort et al. (2005) compared the spore heat resistances of a laboratory strain of *B. subtilis* 168 with that of *B. subtilis* A163 which was isolated from peanut chicken soup, and found significant differences in spore heat resistances, namely a *D*-value of 1.4 min at 105 °C for strain 168 and 0.7 min at 120 °C for strain A163. In a study performed by Lima et al. (2011), spores with high thermal resistance were isolated from cocoa powder. The spores with the highest thermal resistance mainly belonged to the *B. subtilis* group and displayed large variation in spore heat resistance after sporulation under laboratory conditions (Lima et al., 2011).

The observed variations in spore heat resistance within a species can complicate predictive modeling and design of food processes. Therefore, better insight into spore heat resistance is required including the effect of strain variation on spore heat resistance. In addition, most inactivation kinetics are determined in batch heating experiments, thereby complicating the translation of the results to industrial flow inactivation processes such as UHT treatment (Dogan et al., 2009; Wescott et al., 1995; Witthuhn et al., 2011). Only a limited number of studies on spore heat resistance have been performed in continuous flow heating systems. It has been shown that the continuous flow heating system had a higher lethality compared to batch heating for *Bacillus flexus* and *Geobacillus stearothermophilus* (Dogan et al., 2009). Wescott et al. (1995) also reported higher lethality of *B. cereus* spores during continuous flow heating than during batch heating. However, for spores from *G. stearothermophilus*, batch heating was shown to be more lethal compared to continuous flow heating at the tested conditions (Wescott et al., 1995). Clearly, there is a need to establish the effect of inactivation of spores in batch and continuous flow heating for *B. subtilis* spores.

The aim of this study was two-fold, namely, to assess variation in spore heat resistance between strains belonging to the *B. subtilis* group (Vos et al., 2009), and secondly to assess the spore inactivation kinetics in continuous flow heating using a micro heater and compare these with batch heating data using capillary tubes. To this end, detailed spore inactivation kinetics were determined for fourteen strains of the *B. subtilis* group using a batch heating and continuous flow heating.

2. Materials and methods

2.1. Bacterial strains and identification

The strains investigated in this study were twelve industrial isolates, supplied by food manufacturers, and two type strains, namely *B. subtilis* 168 (*Bacillus* Genetic Stock Center (BGSC) 1A700) and the undomesticated strain *B. subtilis* NCIB 3610 (BGSC 3A1). The heat resistance of spores of these strains was initially screened; strains were selected based on these results, to include the largest variation possible (data not shown). All strains belong to the *B. subtilis* group and are listed in Table 1. For the two type strains the isolation source was not clear (Zeigler et al., 2008). Strain A163 is known to form spores with high thermal resistance properties (Cazemier et al., 2001; Kort et al., 2005; Oomes and Brul, 2004; Oomes et al., 2007). Strains 4068, 4069, 4071, 4072, and 4073 correspond with strains CC2, IIC14, CC16, RL45, and MC85 that have been previously described (Oomes et al., 2007). All strains were deposited in the NIZO culture collection and received a unique strain ID. For all industrial isolates a partial 16S rRNA sequence was determined to verify the species level of the strains (Klijn et al., 1991). Following amplification of the partial 16S fragment using PCR, the product was purified and DNA sequencing was performed by Baseclear (Leiden, The Netherlands). The sequences were used as query input for identification against the database of the Ribosomal Database Project (RDP). The designated species names per strain are presented in Table 1. For each strain, the nucleotide sequences were deposited in GenBank under accession numbers KF916630 to KF916641.

2.2. Spore preparation

Spore crops were prepared as described by Schaeffer *et al.* with slight modifications (Schaeffer et al., 1965). In short, the sporulation medium consisted of Nutrient Broth 8 g/L (NB, Difco), supplemented with 1 mM $MgSO_4$, 13 mM KCl, 0.13 mM $MnSO_4$, 1 mM $CaCl_2$, and a final pH of 7.0. For cultivation on plates the medium consisted of Nutrient Agar (NA, Difco) 23 g/L, supplemented with 1 mM $MgSO_4$, 13 mM KCl, 0.13 mM $MnSO_4$, 1 mM $CaCl_2$, with a final pH of 7.0 (NA). The Luria-Broth (LB) medium was inoculated from the −80 °C stocks, and was incubated for 16 h at 37 °C, with shaking at 200 rpm. The overnight cultures were diluted 100 times in sporulation medium and allowed to grow until an $OD_{600\ nm}$ of 0.6,

Table 1
Strains used in this study, with corresponding strain numbers and isolation sources. Per strain the identification based on partial 16s rRNA are indicated and corresponding Genbank accession numbers.

Strain NIZO nr.	Received as	Isolated from	Identification based on 16S	Genbank accession no.	Reference
4062	1A700- type strain 168	Not relevant	Not relevant	Not relevant	BGSC
4060	3A1- type strain NCIB 3610	Not relevant	Not relevant	Not relevant	BGSC
425		Sterilized milk	*B. amyloliquefaciens*	KF916630	This study
4067	A163	Peanut chicken soup (sterilized in can)	*B. subtilis*	KF916631	(Cazemier et al., 2001; Kort et al., 2005; Oomes and Brul, 2004; Oomes et al., 2007)
4068	CC2	Curry cream soup (sterilized in pouch)	*B. subtilis*	KF916632	(Oomes et al., 2007)
4069	IIC14	Binding flour (ingredient)	*B. subtilis*	KF916633	(Oomes et al., 2007)
4071	CC16	Curry cream soup (sterilized in pouch)	*B. subtilis*	KF916634	(Oomes et al., 2007)
4072	RL45	Red Lasagna sauce (pasteurized in glass jar)	*B. subtilis*	KF916635	(Oomes et al., 2007)
4073	MC85	Curry soup (sterilized in glass jar)	*B. subtilis*	KF916636	(Oomes et al., 2007)
4140		Pizza	*B. amyloliquefaciens*	KF916637	This study
4143		Surimi	*B. subtilis*	KF916638	This study
4144		Quiche	*B. vallismortis*	KF916639	This study
4145		Pasta	*B. subtilis*	KF916640	This study
4146		Curry sauce	*B. subtilis*	KF916641	This study

subsequently 200 μL of a culture was spread on three agar plates per strain. The plates were incubated at 37 °C for seven days and spore formation was followed microscopically. The spores were harvested by swabbing the entire bacterial layer of three plates, combined in one tube, and washed by three successive steps in sterile water (5000× *g*, 10 min, 4 °C). The spore suspensions were stored in sterile water at 4 °C for at least one month to allow for spore maturation, before being used in heat-inactivation experiments. Two independent spore crops were prepared for each strain.

2.3. Spore enumeration

To determine the initial spore count, spore suspensions were heated at 80 °C for 10 min to inactivate germinated spores and vegetative cells and to allow for activation of germination. Subsequently, samples were serially diluted in peptone water, and appropriate dilutions were pour-plated in NA in duplicate. Based on the initial spore yields, the spore suspensions were further diluted prior to batch inactivation and continuous flow inactivation experiments, as described below. The number of surviving spores were determined following different heat treatments by serially diluting the samples in peptone water and pour plating appropriate dilutions. All counts were performed after incubation for five days at 37 °C.

2.4. Batch heating inactivation

For each strain, the spore heat inactivation kinetics were determined in a batch heating system using capillary tubes. The experiments were performed twice per strain, using two independent spore preparations. For each spore preparation, the recoveries were determined using three different temperatures, each with at least five different time points. The inactivation kinetics were determined as previously described by Xu et al. (2006). In short, the spore suspensions were diluted to an initial count of approximately 1×10^8 colony forming units per milliliter (CFU/mL, in phosphate buffered saline (PBS), with a pH of 7.4. A capillary tube ($ø_{ext}$ 1.0 mm, $ø_{int}$ 0.8 mm, length 150 mm, catalog no 612-2806, VWR, Amsterdam, The Netherlands) was filled with a spore suspension of 50 μL, which was subsequently heat sealed. Each tube was completely submerged in an oil bath at a selected temperatures for a given time and subsequently transferred to an ice-water bath for 10 min. The sealed capillary tubes were then incubated in a hypochlorite solution (525 ppm) for 10 min and washed with sterile peptone water. The capillary tubes were then transferred to 5 mL sterile peptone water and crushed with a magnetic stirrer by mixing on a vortex. The spores were subsequently enumerated as described before. Using the same method, the initial spore count was determined for each spore suspension following a heat treatment of 80 °C for 10 min, 100 °C for 10 min, and in the absence of a heat activation, to establish optimal germination, which might require heat activation.

2.5. Heat inactivation using continuous flow

For each strain, the continuous flow heat inactivation kinetics of spores were also determined using an in-house continuous flow micro heater, mimicking UHT treatment on a small scale, as described by van der Veen et al. (2009). The micro heater contains a heating-up section in which the spore suspension is quickly heated to the desired temperature using a heat exchanger, a holding section with a fixed length that is submerged in an oil bath set to the desired temperature and a cooling section in which the spore suspension is rapidly cooled using a heat exchanger. The flow rate used for the spore suspension in the micro heater was 5 L/h. At this flow rate, the residence times in the heating-up and cooling section were 1.7 s for each section. The residence times of the spore suspensions during heating in the three different holders with different lengths were 3, 6 and 10 s. The heating experiments were performed for all strains and using one of the two spore crops of each strain. For each experiment, a spore suspension of 5 mL was inoculated in 5 L PBS, resulting in an initial spore count of approximately 1×10^6 CFU/mL, which was determined before each experiment. For each strain the spores were pumped through the micro heater and subjected to different time-temperature combinations: at each of the three set holding times, namely 3, 6 and 10 s, the spores suspensions were subjected to ten different temperatures. After each experiment the temperature of the holding section was lowered by 2 °C (Supplementary Table 3). The viable spore counts before and after each time-temperature combination were determined. In this way, spore suspensions of individual strains were subjected to three set heating times and a temperature range spanning 20 °C, which was selected to result in outcomes ranging from complete inactivation to complete survival, with at least 2 data points showing detectable inactivation. Surviving spores were enumerated as described above.

2.6. Data analysis

For fourteen strains, extensive spore inactivation data were obtained from two independent spore crops using batch heating. The inactivation data were fitted with the log-linear model in Equation (1), to determine the *D*-value, the decimal reduction time, at the corresponding temperature, using Excel. The effective heating times of the spore suspension in the capillary tubes were calculated by taking the total time of submersion in the oil bath minus the modeled time for heating-up and cooling down, which were calculated based on a *z*-value of 10 °C (David and Merson, 1990).

$$\log N(t) = \log N(0) - \frac{t}{D} \tag{1}$$

To determine the temperature dependency of the *D*-value, the *z*-value was determined. The *z*-value was calculated per strain based on the *D*-values of two independent spore crops, as the negative reciprocal of the slope of the plot of log*D* against the temperature, as displayed in Equation (2).

$$z = -1/\text{slope}(\log D, T) \tag{2}$$

The log*D* values of all strains were plotted against the temperature to visualize strain variability. Based on this visualization, two groups of spore heat resistance were identified with log*D* values that cluster together. To compare the two groups with different spore heat resistances, the overall *z*-value, using Equation (2), and subsequently the $\log D_{ref}$ at a reference temperature of 120 °C, were determined per group using Equation (3). Based on the $\log D_{ref}$ at the reference temperature, *D*-values can be estimated at each desired temperature using Equation (4).

$$\log D_{ref} = \text{intercept}(\log D, T) - T_{ref} \Big/ z \tag{3}$$

$$\log D_T = \log D_{ref} - \Big(T - T_{ref}\Big) \Big/ z \tag{4}$$

The 95% prediction interval (PI) of the $\log D_{ref}$ was calculated using the following equation:

$$\log D_{ref} \pm t_{DF,\ 1-0.5\alpha} \sqrt{\frac{\text{RSS}}{\text{DF}}} \tag{5}$$

Where t_{DF} is the student t-value with degrees of freedom (DF), α is the confidence level ($\alpha = 0.05$), and the residual sum of squares (RSS) is calculated from the data points deviating from the regression line.

In the continuous flow system using the micro heater, the temperature is varied while the heating times are fixed. For each heating regime at which heat inactivation occurred a *D*-value was calculated using Equation (1), based on the assumption of log-linear inactivation as determined in batch heating and using the effective heating time. *D*-values that were higher than two times the experimental duration were excluded. By plotting the log*D* values against the temperature, a *z*-value was calculated for each strain using Equation (2). When plotting the log*D* values of all strains, the same two groups of spore heat resistance were identified as in the batch experiments. For both groups a *z*-value and a $\log D_{ref}$ and corresponding upper 95% PI were determined as described above.

2.7. Statistical analysis

An *F*-test was used to test significant differences, based on the plotting of the log*D* values against the temperature. The *F*-test was performed to test if the slope and the intercept of the log*D* of were significantly different. A confidence level of $\alpha = 0.05$ was used. The *F*-test was applied to test whether the two groups of with presumed different spore heat resistances were indeed significantly different. Additionally, the *F*-test was used to test significant differences between the batch- and continuous flow heating, per strain and for the two groups of heat resistant strains.

2.8. Comparison to literature data

Literature data, in the form of *D*-values, was collected from 12 strains belonging to the *B. subtilis* group (Kort et al., 2005; Leguérinel et al., 2007; Lima, 2012; Lima et al., 2011; Nakayama et al., 1996). The *D*-values were log transformed and plotted within the 95% prediction intervals of the two groups of spore heat resistance as determined in the batch heating experiment.

3. Results

3.1. Identification of strains

For the twelve food isolates, the partial DNA sequences of 16S rRNA were determined to identify the strains at the species level. The identification indicated that nine strains belonged to the species *B. subtilis,* two strains belonged to the species *Bacillus amyloliquefaciens* and one strain was identified as *B. vallismortis* (see Table 1).

3.2. Spore heat inactivation kinetics following batch heating

For fourteen strains of the *B. subtilis* group, the thermal inactivation kinetics were determined. The survival plots showed straight lines, without tailing (data not shown), based on at least 5 different time points per heating temperature. The inactivation plots were fitted with the log-linear inactivation model, by which the different *D*-values were determined per strain per temperature. The *D*-values ranged from $D_{100\ ^\circ C}$ of 1.15 min for strain 4144 to $D_{125^\circ C}$ of 0.53 min for strain 4067 as displayed in Table 2. Biological variation between the different spore crops of the same strain was observed. The *D*-values per strain were log transformed and plotted against the temperature to determine the *z*-values per strain, based on two independent spore batches. The *z*-values ranged from 5.82 °C (Standard Error (S.E.) ±0.38 °C) for strain 4073 to 8.32 °C (±0.93 °C) for strain 4144 (Table 2). The regression coefficients of the *z*-value estimation ranged from 0.90 to 1.00.

Thereafter the log*D* values of all strains together were plotted against the temperature to visualize strain variation. Based on this visualization, spore heat resistance could be grouped in two clusters, as presented in Fig. 1A. Strains 4060, 4062, 4140, 4143, 4144 belonged to the low spore heat resistance group, whereas strains 425, 4067, 4068, 4069, 4071, 4072, 4073, 4145, and 4146 all belonged to the high spore heat resistance group. The variation within the lower resistant group was smaller compared to the variation within the high heat resistant group. The slopes of the two groups of spore heat resistance were not significantly different ($p = 0.09$), whereas the intercepts of the two groups were significantly different ($p < 0.0001$). The calculated *z*-values for the low heat resistant group was 7.6 °C (±0.4 °C) and the *z*-value for the high heat resistant group was 9.5 °C (±0.8 °C). For both groups the $\log D_{ref}$ and corresponding upper 95% upper prediction interval, were calculated (Table 3) and corresponding $D_{120\ ^\circ C}$, which was 0.34 s (upper 95% PI = 0.74 s) for the low spore heat resistance group and 45.7 s (upper 95% PI = 242 s) for the high spore heat resistance group. Plotting the literature data of 12 strains belonging to the *B. subtilis* group (Supplementary material 1), displayed that most data points fell within the 95% PI of the two groups of spore heat resistance identified in this study.

3.3. Spore heat inactivation kinetics following continuous flow heating

For the analysis of the flow heating data, a *D*-value was calculated for each data point where inactivation occurred (Supplementary material 2). The *z*-values were calculated per strain and ranged from 6.03 °C (±0.83 °C) for strain 4140 to 15.84 °C (±3.77 °C) for strain 4069. The regression coefficients for the *z*-value ranged from 0.50 to 0.90, indicating a large variation in the goodness of the fit. The plotting of all the log*D* values against the temperature showed the same separation in two groups of spore heat resistance as observed in the batch heating experiment, with both groups encompassing the same strains (Fig. 1B). The slopes of the plotted log*D*-values, and thus the *z*-value, did not differ significantly between the two groups ($p = 0.068$), while the intercept was again found significantly different ($p < 0.0001$). The calculated *z*-value for the low spore heat resistance group was 12.7 °C (±1.8 °C), whereas the *z*-value for the high heat resistance group was 18.3 °C (±2.2 °C). The corresponding regression coefficients were 0.55 and 0.47 for the low and high spore heat resistance groups, respectively. For both groups the $\log D_{ref}$ and corresponding upper 95% upper prediction interval were calculated (Table 3), and corresponding $D_{120\ ^\circ C}$ which was 0.07 s (upper 95% PI = 2.34 s) for the low spore heat resistance group and 8.5 s (upper 95% PI = 26.9 s) for the high spore heat resistance group.

3.4. Spore inactivation during batch and continuous flow heating

Per strain the plotted log*D* values of the batch heating and the continuous flow heating were tested for significant differences in the slope and the intercept (Table 2). The slopes of the plotted log*D* values, signifying the *z*-value, during batch heating and continuous flow heating did not differ significantly for eight strains. Additionally, from these eight strains, for six strains the intercept did not differ significantly. For the other six strains, the slopes of the plotted log*D* values during batch heating and continuous flow heating differed significantly, and the *z*-value was higher in flow heating compared to batch heating. For the groups with either low or high spore heat resistances, the slopes of the plotted log*D*-values differed significantly when comparing the batch- and the

Table 2
The calculated *D*-values per strain for the independent spore crops, at three different temperatures. The calculated *z*-value for each strain as determined using batch and continuous flow heating. The F-test, to test for significant differences in the slope and the intercept of the plotted log*D* values, between the different heating methods used of the per strain.

Strain	Temperature (°C)	Batch heating									Flow heating			*F*-test	
		Spore crop 1			Spore crop 2						*z*-value (°C)	S.E	r^2	Significant difference	
		D (min)	S.E	r^2	*D* (min)	S.E.	r^2	*z*-value (°C)	S.E	r^2				Slope	Intercept
4062	100	3.53	0.21	0.96	2.97	0.22	0.94	6.88	0.37	0.99	6.40	0.96	0.88	No	No
	105	0.65	0.08	0.91	0.84	0.06	0.90								
	110	0.10	0.02	0.67	0.13	0.01	0.88								
4060	100	4.39	0.38	0.90	2.93	0.42	0.91	7.53	0.58	0.98	7.76	1.03	0.90	No	No
	105	0.83	0.09	0.90	0.65	0.06	0.91								
	110	0.21	0.02	0.90	0.13	0.02	0.82								
425	110	12.59	0.79	0.97	8.23	0.41	0.97	6.23	0.41	0.98	7.61	1.34	0.78	No	No
	115	2.36	0.17	1.00	1.56	0.08	0.99								
	120	0.28	0.01	0.98	0.23	0.01	0.99								
4067	115	18.14	1.93	0.89	10.24	0.61	0.94	6.31	0.66	0.96	15.57	2.66	0.77	Yes	N.T.[a]
	120	1.79	0.07	0.99	1.58	0.05	0.99								
	125	0.24	0.01	0.99	0.53	0.05	0.94								
4068	115	4.64	0.40	0.96	5.81	0.53	0.94	6.58	0.31	0.99	12.55	5.10	0.50	No	No
	120	0.66	0.02	0.98	0.79	0.04	0.98								
	125	0.15	0.00	0.99	0.16	0.02	0.92								
4069	115	3.73	0.21	0.95	2.63	0.15	0.96	7.29	0.35	0.99	15.84	3.77	0.72	Yes	N.T.
	120	0.57	0.02	0.98	0.58	0.06	0.96								
	125	0.12	0.01	0.92	0.14	0.01	0.93								
4071	110	18.64	2.48	0.83	10.36	0.53	0.98	6.68	1.03	0.91	12.94	1.50	0.90	Yes	N.T.
	115	1.18	0.08	0.88	1.31	0.08	0.92								
	120	0.65	0.03	0.97	0.30	0.04	0.77								
4072	115	3.14	0.27	0.91	2.66	0.14	0.93	7.80	0.66	0.97	10.96	3.47	0.67	No	No
	120	0.99	0.05	0.96	0.60	0.02	0.96								
	125	0.12	0.01	0.96	0.18	0.02	0.90								
4073	115	9.64	0.92	0.92	4.77	0.64	0.90	5.82	0.38	0.98	13.59	4.55	0.82	Yes	N.T.
	120	0.92	0.03	0.98	0.79	0.04	0.97								
	125	0.13	0.01	0.93	0.13	0.01	0.96								
4140	100	3.30	0.36	0.94	3.32	0.64	0.85	7.21	0.17	1.00	6.03	0.83	0.91	No	No
	105	0.60	0.06	0.96	0.58	0.09	0.95								
	110	0.13	0.02	0.98	0.14	0.02	0.94								
4143	100	3.47	0.14	0.99	1.62	0.13	0.93	7.50	1.22	0.90	13.74	3.70	0.73	No	No
	105	0.85	0.07	0.99	0.80	0.10	0.75								
	110	0.17	0.02	0.91	0.07	0.01	0.87								
4144	100	1.73	0.24	0.88	1.15	0.13	0.91	8.32	0.93	0.95	12.34	2.36	0.77	No	No
	105	0.54	0.14	0.74	0.25	0.04	0.87								
	110	0.08	0.01	0.91	0.09	0.01	0.96								
4145	115	17.64	1.99	0.91	9.15	0.33	0.97	6.09	0.56	0.97	10.10	1.11	0.88	Yes	N.T.
	120	2.96	0.36	0.92	1.52	0.07	0.99								
	125	0.26	0.02	0.97	0.33	0.01	0.99								
4146	105	22.83	1.94	0.92	9.27	0.55	0.95	6.90	0.70	0.96	11.56	2.18	0.85	Yes	N.T.
	110	3.03	0.10	0.99	2.25	0.13	0.89								
	115	0.56	0.03	0.95	0.48	0.02	0.97								

[a] N.T. = Not tested.

continuous flow heating system. The *z*-value was generally higher in the flow heating system than in the batch heating system.

4. Discussion

In this study we establish that spores of different *B. subtilis* group isolates display highly significant differences in heat resistance. Two distinct groups could be identified based on a thorough analysis of the spore heat resistances of fourteen strains, using a wide range of time-temperature combinations for heat exposure. This study thus provides a detailed description of variation in spore heat resistance of *B. subtilis* group strains, and renders a modeling approach using two spore inactivation kinetics for highly heat resistant strains versus lower heat resistant strains. The spore heat resistance varied from a $D_{120\ ^\circ C}$ of 0.34 s for the low spore heat resistance group to a $D_{120\ ^\circ C}$ of 45.7 s for the high spore heat resistance group, thus a factor 130 different. Moreover, spore heat resistance is commonly determined under laboratory conditions using batch heating systems, while in industry, continuous flow heat inactivation is widely applied, often using higher temperatures and shorter heating times, leaving the question whether extrapolation of batch data to flow data is justified. In this study, the inactivation kinetics for fourteen strains of *B. subtilis* group showed significant differences in the *z*-value between batch heating and continuous flow heating, hence extrapolation from one heating system to the other is not justified. While the heating systems have an influence on the efficacy of spore inactivation, overall, the impact of strain variability was much greater than the impact of this variable.

Based on spore heat resistance, strains of the *B. subtilis* group could be grouped into two clusters when plotting the log*D* values against temperature. This holds true for both the batch inactivation data and the continuous flow inactivation data, and in both cases, the same strains clustered together. Variation in spore heat resistance of strains within the *B. subtilis* species and *B. subtilis* group has been reported before (Lima et al., 2011; Oomes et al., 2007). For *Clostridium perfringens*, strain variation in spore heat resistance was observed with varying $D_{90\ ^\circ C}$ values

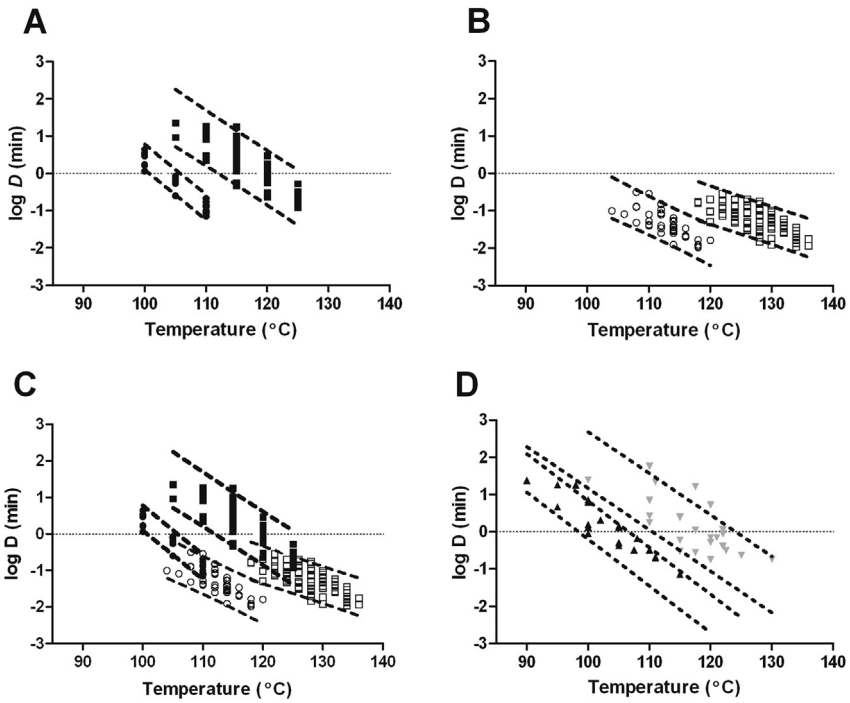

Fig. 1. Plot of the estimated log*D* values, plotted against the temperature of fourteen strains of the *B. subtilis* group and corresponding 95% prediction intervals, determined in capillary tubes (A), determined in a micro-heater (B), and the combined data sets (C). The literature log*D* values were plotted in the 95% prediction interval of the batch heating (D) The symbol ● represents the data points from the lower spore heat resistance group and ■ represents the data points of the higher spore heat resistance group, determined using batch heating. The ○ symbol represents the data points from the low spore heat resistance group and □ represents the data points from the high spore heat resistance group, determined in the micro-heater. The symbol▲ represents low spore heat resistance data and ▼ represents high spore heat resistance data from literature.

ranging from 5.5 min to 120.6 min (Orsburn et al., 2008). In addition for *B. cereus*, where spore inactivation kinetics were globally assessed, strain variation was identified as significant factor (van Asselt and Zwietering, 2006). The high number of strains used in the current study allowed for a statistical analysis that rendered two groups with respect to spore heat resistance. In our analysis, strain *B. subtilis* A163 was incorporated (corresponding with strain nr. 4067) and showed $D_{120\ ^\circ C}$ values of 1.79 and 1.53 min, which is higher than the previously reported $D_{120\ ^\circ C}$ of 0.7 min by Kort et al. (2005). However, the reported *z*-value of 6.1 °C for this strain (Kort et al., 2005) was similar to the z-values found in this study, *i.e.* 6.3 °C (±0.7 °C). A possible explanation for the difference in $D_{120\ ^\circ C}$-value is the different preparation method of spores, on plates in this study, and in liquid medium for the other experiment (Kort et al., 2005). The phenomenon that spores produced on surfaces are more heat resistant than spores produced from planktonic cells in liquid media has previously been reported by Rose et al. (2007), who observed higher spore heat resistances for *B. subtilis* when spores were prepared following growth on agar plates compared with liquid medium. There are multiple other factors known to contribute to the final spore heat resistance. Generally the higher the sporulation temperature, the higher the final spore heat resistance properties (Nicholson et al., 2000). The sporulation of *B. subtilis* in a natural or a processing environment might occur in biofilms, and complex colony growth allows the formation of more heat resistant spores (Lindsay et al., 2006; Veening et al., 2006). The composition of the sporulation medium is also important, including different salts added to the medium, such as magnesium, manganese, potassium, and in particular calcium, are known to increase the final heat resistance of spores of *B. subtilis* (Cazemier et al., 2001; Oomes and Brul, 2004; Oomes et al., 2009). Calcium is also required for a spore to reach full heat resistance, after release from the mother cell, in the maturation process (Sanchez-Salas et al., 2011). In this study, the spores of all strains were allowed to form, and mature under the same conditions, to rule out the effect of variation in sporulation conditions on spore heat resistance. No variations in sporulation conditions were applied; the observed differences in spore heat resistance between strains are thus a specific property of the strain. A generally observed phenomenon is that the spore heat resistance decreases after re-sporulation under laboratory conditions (Lima et al., 2011; van Zuijlen et al., 2010). It is important to consider that the exact history of spores encountered in food matrices, such as the sporulation and maturation conditions, is not known. The points obtained by plotting of the literature data fell mainly within the prediction intervals of the two groups of spore heat resistance. This suggests that variation in spore heat resistance is strain specific, since the literature data originated from strains that were sporulated under different conditions.

Table 3
The calculated *z*-values and logD_{ref} for the two groups of spore heat resistance, for batch heating and continuous flow heating.

Spore heat resistance group	Heating method	*z*-value (°C)	S.E.	r^2	log*Dref* (min)	Upper 95% PI	$D_{120\ ^\circ C}$ (s)	*n*
Low spore heat resistance	Batch	7.5	0.4	0.92	−2.24	−1.91	0.34	30
	Flow	12.7	1.8	0.55	−1.92	−1.41	0.72	40
High spore heat resistance	Batch	9.3	0.8	0.72	−0.12	0.61	45.7	54
	Flow	18.3	2.2	0.47	−0.85	−0.35	8.5	81

In continuous flow heating using the micro heater the main variable factor was the temperature, with fixed times. The time-temperature combinations in continuous flow heating consisted of relatively short times and high temperatures, while heat exposure in batch heating was characterized by longer heating times and somewhat lower temperatures. For eight strains there was no significant difference in the slope of the plotted log*D* values, signifying the *z*-value, comparing batch- and continuous flow heating. However, for two out of the eight strains, the intercept, signifying the spore heat resistance, did differ significantly. For the other six strains, the *z*-value was significantly higher determined in continuous flow heating compared to batch heating. Thus, the justification of extrapolation from one heating system to the other, varies from strain to strain within the *B. subtilis* group.

Additionally, for the two groups of spore heat resistance that clustered together, in both cases the slopes of the plotted log*D* values, signifying the *z*-value, were significantly different when comparing batch and continuous flow heating. For the two groups of spore heat resistance, extrapolation from one heating system to the other is not justified. The *z*-value was higher when determined in the continuous flow heating, and thus at higher temperatures. This is consistent with the finding that at higher temperature ranges, generally higher *z*-values were observed (Edwards et al., 1965). Dogan et al. (2009) observed a higher lethality for continuous flow heating compared to batch heating for *B. flexus* and *G. stearothermophilus* spores. In accordance, Wescott et al. (1995) determined a higher lethatlity for continuous flow heating for spores of *B. cereus* compared to batch heating. However, in contrast to the results from this study, for *G. stearothermophilus* a higher lethality for batch heating was observed compared to continuous flow heating. In this study significant differences in the *z*-value were identified, therefore the difference in lethality would depend on the time-temperature combination selected. To globally assess the impact of variation in the *z*-value on inactivation kinetics, the $D_{100\,^{\circ}C}$ and $D_{145\,^{\circ}C}$ were calculated for the two groups of spore heat resistance and for the two different heating systems. Due to the difference in *z*-value, the inactivation of spores from stains in both the low and high spore heat resistance groups was more efficient in continuous flow heating than in batch heating at 100 °C, whereas batch heating was more efficient than continuous flow heating at 145 °C. When designing a heat inactivation process it is important to consider the two groups of spore heat resistance and the variation in *z*-value among strains and between the different heating methods.

Multiple time-temperature combinations can be proposed, based on batch heating in capillary tubes, to distinguish the two groups of spore heat resistance within the *B. subtilis* group. Heating for one hour at 100 °C, will result in a 10.2 log reduction for the low spore heat resistance group and a 0.1 log reduction for the high spore heat resistance group, using the logD_{ref} from the batch heating experiment. Using a similar approach, heating for 5 min at 110 °C will result in a 18.6 log reduction for the low spore heat resistance group, and a 0.1 log reduction for the high spore heat resistance group. It should be noted that the proposed time-temperature combinations are based on spores prepared under laboratory conditions, and do not include variations in spore heat resistance based on the history of the spores and a potential effect of the food matrix.

5. Conclusions

In this study the spore heat inactivation kinetics were determined in detail for fourteen stains belonging to the *B. subtilis* group. Two distinct groups of spore heat resistance were identified, with batch heating using capillary tubes, and with continuous flow heating using a micro-heater. The spore heat resistance within *B. subtilis* can be separated in two groups, suggesting that spore heat resistance is not a species, but rather a strains specific property.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2014.04.009.

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