International Journal of Food Microbiology 291 (2019) 161–172

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Heat resistance of spores of 18 strains of *Geobacillus stearothermophilus* and impact of culturing conditions

Marjon H.J. Wells-Bennik[a,c], Patrick W.M. Janssen[a,c], Verena Klaus[a,c], Chi Yang[a,c], Marcel H. Zwietering[b,c], Heidy M.W. Den Besten[b,c,*]

[a] NIZO, 6718 ZB Ede, the Netherlands
[b] Laboratory of Food Microbiology, Wageningen University, 6700 AA Wageningen, the Netherlands
[c] Top Institute Food and Nutrition, 6709 PA, Wageningen, the Netherlands

ARTICLE INFO

Keywords:
Sporulation
Germination
Inactivation
Enumeration
Variability
Modelling

ABSTRACT

In this study, different methods were evaluated for enumeration of spores of *G. stearothermophilus*, different sporulation methods were assessed for yields and wet heat resistances of obtained spores, and subsequently, the variation in heat resistances of spores was determined. Overall, tryptone soya agar (TSA) was the most suitable medium for enumeration of spores of this thermophilic bacterium. Sporulation on different media both at 55 and at 61 °C led to considerable variation in spore heat resistance. The heat resistance of spores was highest upon sporulation on medium supplemented with free ions of calcium, potassium, magnesium and manganese (CaKMgMn). For 18 different *G. stearothermophilus* strains that were isolated from various sources, spores were subsequently produced on nutrient agar supplemented with CaKMgMn at 55 °C. Strain ATCC 12980^T, also known as 9A20, which is commonly used in steam sterilization tests was included. The survival of spores of all strains was assessed at 125 °C and 130 °C using two independent spore batches per strain. The mean $D_{125^\circ C}$ for spores of the 18 strains was 1.1 min (95% PI 0.48–2.3 min) and the mean $D_{130^\circ C}$ was 0.37 min (95% PI 0.17–0.82 min). For spore inactivation of these 18 strains, a *z*-value of 11.1 °C was estimated, resulting in an estimated *D*-value of 2.4 min (95% PI 1.1–5.2) at the reference temperature 121.1 °C. Based on the data sets obtained in this study, it was found that the variability in spore heat resistance could largely be attributed to strain variability and conditions used during sporulation (especially the sporulation medium); reproduction and experimental variabilities were much smaller. The established variabilities were compared with the overall variability in spore heat resistance of *G. stearothermophilus* based on a meta-analysis of reported *D*-values. The data presented indicate that strain variability and history of sporulation each account for approximately half of the overall variability observed with respect to the heat resistance of spores of *G. stearothermophilus*. The findings presented in this study allow for optimal recovery of *G. stearothermophilus* spores from foods and a better understanding of factors that determine the heat resistance properties of spores of *G. stearothermophilus*. Moreover, this study once more underlines the limited effects of heat treatments used in the food industry on inactivation of spores of this bacterium.

1. Introduction

The genus *Geobacillus* comprises a group of spore formers that are obligately thermophilic and which produce thermoresistant spores. The growth temperature range of *Geobacillus* species is 37–75 °C, with an optimum at 55–65 °C (Nazina et al., 2001). *Geobacillus* spp. are aerobic or facultative anaerobic and use oxygen as the electron acceptor, replaceable in some species by nitrate (Downey et al., 1969; Nazina et al., 2001). Spores, including those of *G. stearothermophilus,* are ubiquitously found in nature (Postollec et al., 2012; Scheldeman et al., 2005; Zeigler, 2001) and may contaminate foods and food ingredients. This may be problematic in the production of commercially sterile food products, as some spores are able to survive industrial sterilization processes and ultra high temperature (UHT) treatments (134–145 °C, for 1–10 s) (André et al., 2013; Burgess et al., 2010; Curran and Evans, 1945; Schwarzenbach and Hill, 1999; Wells-Bennik et al., 2016; Wescott et al., 1995; Westhoff and Dougherty, 1981).

In addition, the organism may grow and form spores during food manufacturing, particularly when temperatures during processing are elevated (e.g. 50 to 60 °C) for prolonged periods of times (Burgess et al.,

* Corresponding author at: Laboratory of Food Microbiology, Wageningen University, 6700 AA Wageningen, the Netherlands.
E-mail address: heidy.denbesten@wur.nl (H.M.W. Den Besten).

https://doi.org/10.1016/j.ijfoodmicro.2018.11.005
Received 6 April 2018; Received in revised form 14 October 2018; Accepted 6 November 2018
Available online 10 November 2018

2010; Murphy et al., 1999; Scott et al., 2007). Thermophilic spores are frequently found in milk powders at levels that can exceed 10^3 spores/g (Oomes et al., 2007, Rückert et al., 2004; Watterson et al., 2014; Yuan et al., 2012).

Spores of *G. stearothermophilus* that survive heat treatments may germinate in finished product, and emerging vegetative cells may multiply and lead to spoilage when the product is exposed to elevated temperatures in the distribution chain, with reported temperatures for growth of 37–70 °C for *G. stearothermophilus* (Claus and Berkeley, 1986; Durand et al., 2015). In addition, intrinsic properties of the food should allow for growth of the organism. Most strains do not grow at NaCl concentrations of > 4% (Durand et al., 2015), but growth has been reported at salt concentrations of up to 5% NaCl (Nazina et al., 2001). The pH required for growth has been reported as 6.0–8.5 with optimal values between 6.2 and 7.5 (Claus and Berkeley, 1986; Nazina et al., 2001), but Durand et al. (2015) found that certain strains are able to grow at pH values as low as 5.0. The organism is frequently implicated in cases of 'flat-sour' spoilage of heat treated foods (André et al., 2013), resulting from conversion of saccharides to organic acids without gas formation (Kalogridou-Vassiliadou, 1992). In addition, the bacterium is able to produce lipases and proteases (Chen et al., 2004), which may lead to spoilage and off-flavours, and is able to reduce nitrate at reduced oxygen tension (Downey et al., 1969; Ho et al., 1993).

Various methods are available to enumerate different types of spores in food ingredients and foods, generally consisting of a heat treatment followed by plating that allows for germination of spores and outgrowth. Different methods will have different outcomes, as was clearly demonstrated by Kent et al. (2016). For enumeration of aerobic spores, generally, a heat treatment of 10 min 80 °C is applied to eliminate vegetative cells prior to plating (ISO 6730:2005; NEN 6813:2014; Stevenson and Lembke, 2015). For the enumeration of high level heat resistant spores of thermophiles, a much higher initial heat treatment is applied of 30 min 100 °C (NEN 6809:2014) followed by plating and incubation at 55 °C. In the case of enumeration of especially heat resistant spores in milk powders, an even higher initial heat treatment of 30 min at 106 °C is used (ISO/TS 27265:2009). Such heat treatments will favour the recovery of spores with high level heat resistance and inactivate spores that are less heat resistant (Den Besten et al., 2018).

For optimal recovery and enumeration of spores of *G. stearothermophilus* using culturing techniques, a cultivation medium should be selected that supports germination and growth to visible colonies. It is known that the choice of medium can affect the spore recovery of *G. stearothermophilus* (Cook and Gilbert, 1968a; Dlugokenski et al., 2011; Mallidis and Scholefield, 1986; Pflug et al., 1981). For instance, Dextrose Tryptone Agar (DTA) gave 2-fold lower recoveries than AAMS (Cook and Gilbert, 1968a), and media obtained from different suppliers, or distinct batches of a certain brand from the same supplier, can lead to different recoveries (Pflug et al., 1981). In addition, agar media with pH < 6.6 or NaCl concentrations > 0.9% may negatively affect recovery of heat treated spores (Cook and Brown, 1964; Feeherry et al., 1987; López et al., 1997; Yazdany and Lashkari, 1975). Detection of *G. stearothermophilus* spores in foods may also be complicated by the low concentrations present, the presence of food particles which may interfere with colony counting, and the presence of thermoresistant spores of other thermophilic species that outcompete *G. stearothermophilus* (Kent et al., 2016). Thus, it is important to apply an enumeration method that favours outgrowth of *G. stearothermophilus*, and apply an appropriate heat treatment prior to plating to inactivate competitive bacteria that produce spores that are less heat resistant. Lastly, the incubation temperature can affect spore recovery of *G. stearothermophilus*, with optimal temperatures of 50 to 55 °C (Cook and Gilbert, 1968a; Feeherry et al., 1987; López et al., 1997).

For *Bacillus* species, it is known that strain variability can have a major effect on spore heat resistance (Berendsen et al., 2015, 2016a, 2016b; Den Besten et al., 2017, 2018). The variation in heat resistance of spores of *G. stearothermophilus* is also considerable, as reported by Rigaux et al. (2013). Heat resistance of spores of this species has been reported to be influenced by sporulation conditions, such as temperature and cultivation medium (Cook and Gilbert, 1968a, 1968b; Guizelini et al., 2012; Leguérinel et al., 2007; López et al., 1997; Mallidis and Scholefield, 1986; Nguyen Thi Minh et al., 2011; Williams and Robertson, 1954). Notably, the presence of free ions of calcium, magnesium and manganese in the sporulation medium affects the heat resistance of spores (Cazemier et al., 2001; Oomes and Brul, 2004). However, so far, it is not entirely clear which factors contribute most to heat resistance of spores of this bacterium.

Spores of various strains of *G. stearothermophilus* are being used as biological indicators to evaluate the efficacy of industrial sterilization processes (Sasaki et al., 2000; Zeigler, 2001). However, no studies are available in which the heat resistances of such strains have been compared directly with isolates from foods using spores produced under the same conditions. Such information is important when preparing spores for sterilization validation studies.

The aims of this study were i) to evaluate the recovery of spores of *G. stearothermophilus* on commonly used enumeration media, ii) to determine the yield of spores and their heat resistance using different sporulation methods, and iii) to estimate the impact of various known factors on spore heat resistance. Thereto, the experimental, reproduction, strain and sporulation history (sporulation temperature and medium used) variabilities were determined and compared with the overall variability as found in literature, providing insight in the factors that contribute most to heat resistance of spores of *G. stearothermophilus*.

2. Material and methods

2.1. *Geobacillus stearothermophilus* strains

In total, 18 strains of *G. stearothermophilus* were used in this study. This included type strain 9A20 from the Bacillus Genetic Stock Center (BGSC) (same as ATCC type strain 12980), strain BGSC 9A2 (NRRL B-4419) and various other isolates from food and other environmental sources. A NIZO number was assigned to each strain, and the original numbers, isolation sources and references are listed in Table 1. Stocks of spores of each culture were kept at −80 °C in water plus 20% glycerol.

2.2. Spore production under different conditions

Conditions during sporulation may influence spore yields and heat resistance of spores. For two strains (namely, 9A20 and 4161), spores were produced using eight different media and two temperatures, with the aim to assess the influence of media and incubation temperature on spore yields and spore heat resistance. The media used were Milk agar, Nutrient Agar supplemented with Ca^{2+} K^{+} Mg^{2+} and Mn^{2+} ions (NA CaKMgMn), Nutrient Agar supplemented with Ca^{2+} and Mn^{2+} ions (NA CaMn), Plate Count Agar with 5% skim milk powder (PCA5M), Tryptone Soya Agar (TSA), TSA supplemented with Ca^{2+} K^{+} Mg^{2+} and Mn^{2+} ions (TSA CaKMgMn), yeast extract/beef extract/peptone medium (YBP), and yeast extract/meat extract medium supplemented with $MnSO_4$ (YMMn) (compositions listed in Supplementary Table 1). The incubation temperatures used were 55 and 61 °C (both values are around the optimum growth temperature of *G. stearothermophilus*). Sporulation was achieved by spread plating 200 μL aliquots of overnight (ON) liquid cultures of strains 9A20 and 4161 onto the eight different sporulation agar media. The ON cultures in broth were obtained as follow: using a sterile 10 μL loop, an aliquot from the −80 °C stock was inoculated in 10 mL of Tryptone Yeast Broth (TYB) containing 10 g/L BD Bacto™ Tryptone (Becton Dickinson B.V., Breda, The Netherlands), 5 g/L yeast extract (BD) and 5 g/L NaCl (Sigma-Aldrich, Zwijndrecht, The Netherlands) and incubation was performed at 55 °C with shaking (200 rpm). Subsequently, 200 μL aliquots of the ON cultures were spread plated on three replicate plates per medium and

Table 1
Geobacillus stearothermophilus strains used in this study.

NIZO strain number	Original Nr	Strain information	Reference
9A2	NRRL B4419	From the strain collection of John R. Gillis at the American Sterilizer Co. in Erie, PA; used as a sterilization standard	(Zeigler, 2001)
9A20	ATCC 12980, type strain	Strain 26 from the collection of the National Canning Association. Used in steam sterilization tests	(Zeigler, 2001)
469	C953; DSM 1550	Evaporated milk DSMZ	(Rückert et al., 2004)
4111	T4	Pea soup (8 h 40 °C)	(Oomes et al., 2007)
4112	T26	Pea soup (12 h 60 °C)	(Oomes et al., 2007)
4161	TIFN-09 008	Dairy product	(Zhao et al., 2013)
4163	TIFN-09 035	Used in beta lactam assay in milk testing	(Curran and Evans, 1945)
4225	O15754	Evaporated milk	NIZO culture collection
4309	DSMZ 456	Sugar beet juice from extraction installations	German collection of microorganisms and cell cultures (DSMZ)
4310	DSMZ 457	Sugar beet juice from extraction installations	DSMZ
4312	DSMZ 494	Steam sterilization control	DSMZ
4313	DSMZ 2027; ATCC 7954; ATCC 29609	Canned peas	DSMZ
4314	DSMZ 5934; ATCC7953	Steam sterilization control	DSMZ and described in Sasaki et al. (2000)
4315	CTCPA 2804 166	White beans with goose fat	CTCPA culture collection
4316	CTCPA 2804 111	Fish pie	CTCPA culture collection
4317	CTCPA 2804 081	Mushrooms	CTCPA culture collection
4318	CTCPA 2804 135	Green beans	CTCPA culture collection
4319	CTCPA 2804 158	Corn	CTCPA culture collection

temperature condition, and plates were incubated for 5 days at 55 ± 0.3 °C or at 61 ± 0.7 °C (hereafter referred to as 55 °C and 61 °C, respectively). During incubation, plates were placed inside plastic bags containing a wet tissue, to minimize dehydration of the agar. After 5 days of incubation, spores from three replicate plates were harvested by adding approximately 1 mL of cold sterile water to the surface of each plate and removing the bacterial lawn using a sterile plastic spreader. Spore suspensions were pooled and washed three times in cold sterile water by centrifugation (5000 ×*g*, 10 min, 4 °C).

To simulate industrially relevant sporulation conditions in the dairy industry, two of the eight media used during sporulation were milk-based agars (PCA5M and milk agar) (see above). In addition, a milk-biofilm system was used to produce spores, consisting of sterile metal coupons (stainless steel type 304) that were inserted into tubes (50 mL sterile tube; Greiner, Alphen aan den Rijn, the Netherlands) and partially submerged in 5 mL skimmed UHT-treated milk. Spores were recovered from the surface of coupons and from the liquid phase. A detailed procedure is presented as a footnote to Supplementary Table 1.

Spore suspensions of *G. stearothermophilus* were prepared in sterile water because of the reported negative effect of phosphate on recovery and heat resistance of *G. stearothermophilus* spores (Cook and Gilbert, 1968b; Finley and Fields, 1962; Gauthier et al., 1978; López et al., 1997). Spore suspensions were stored at 4 °C for at least one month before use in heat inactivation experiments.

2.3. Outgrowth on different media

The ability of spores of *G. stearothermophilus* to germinate and form visual colonies was assessed on eight different cultivation media, namely, Antibiotic Assay Medium with Starch (AAMS), Brain Heart Infusion Agar with vitamin B12 (BHI-A B12), Nutrient Agar (NA), Plate Count Milk Agar according to ISO/TS 27265:2009 (PCMA ISO), Starch Agar (SA), Tryptose Blood Agar Base (TBAB), TSA, and TSA supplemented with Ca^{2+} ions (TSA Ca) (abbreviations and compositions listed in Supplementary Table 2). Most of the media used for enumeration were different from the media used to produce spores (as described in Section 2.2), because conditions that favour sporulation and the production of heat resistant spores can be different from the ones that favour outgrowth. The recovery of spores on different cultivation media was assessed using spores of strains 4161 and 9A20 that were obtained upon sporulation on Nutrient Agar supplemented with calcium, potassium, magnesium and manganese (NA CaKMgMn) (Supplementary Table 1) at 55 °C for 5 d (rendering high concentrations of heat resistant spores). Prior to plating to determine spore counts in the spore suspensions, spores were heat treated for 10 min at 99.9 °C (rounded to 100 °C) using a PCR machine (GeneAmp PCR System 9700, Applied Biosystems) (rounded to 100 °C). A relatively high heat treatment was used to inactivate vegetative cells and because it has been demonstrated to result in optimal recovery of spores of *G. stearothermophilus* as shown by Cook and Gilbert (1968a). In addition to the 10 min 100 °C heat treatment, spores were therefore also heated for 90 s at 125 °C using the capillary tube method (described in following section) prior to plating. Serial dilutions of spore suspensions were pour-plated using the eight different media (Supplementary Table 2) and plates were incubated at 55 °C for 5 d in a plastic bag containing a wet tissue.

Besides assessing germination of spores and subsequent outgrowth to visible colonies of strains 4161 and 9A20 on these eight media, vegetative cultures of six other *G. stearothermophilus* strains (namely, 4109, 4111, 4112, 4114, 4163 and 9A2) were streaked on these media to assess their ability to support growth. To this end, individual cultures that were grown overnight to stationary phase in TYB at 55 °C (200 rpm) were streaked on the different media (Supplementary Table 2). Plates were incubated at 55 °C in a plastic bag containing a wet tissue and growth (presence of visual colonies) was assessed after 3 and 6 days. Non-inoculated plates were used as negative controls (no growth detected).

2.4. Assessment of heat resistance of spores produced on different media

The heat resistances of spores of strains 4161 and 9A20 produced on the eight different sporulation media at two different temperatures (55 °C and 61 °C) were determined at temperatures above 100 °C using the capillary tube method which has been described by Xu et al. (2006). Capillary tubes (Øext 1.0 mm, Øint 0.8 mm, length 150 mm, VWR) were filled with 50 μL spore suspension and heat sealed. Heat treatment was performed in a thermostated oil bath by submerging the capillary tubes completely in the oil at the selected temperature for the desired time period. After heating, the capillary tubes were immediately cooled in an ice-water bath for 10 min, then transferred to a hypochlorite solution (525 ppm) for another 10 min and rinsed with sterile water. The glass capillary tubes containing the 50 μL spore suspensions were then placed in tubes containing 5 mL sterile water and a sterile magnetic stirrer bar, and capillary tubes were crushed thoroughly by mixing using a vortex. The concentrations of viable spores were determined by pour plating 1 mL of the suspensions of these tubes in TSA (Tritium Microbiologie BV, Eindhoven), and 10-fold serial dilutions that were made in sterile de-mineralized water. TSA was used as recovery medium when determining spore heat inactivation kinetics of strains 4161 and 9A20 as TSA supported germination and outgrowth of spores of strains 4161 and 9A20 and vegetative growth of strains 4109, 4111, 4112, 4114, 4163 and 9A2 (as described in the Results Section). Plates were incubated at 55 °C for 5 days and subsequently counted to determine the spore concentrations.

2.5. Preparation of highly heat resistant spores of 18 strains

Spores of all 18 *G. stearothermophilus* strains were prepared using the procedures that rendered spores with the highest heat resistances in our experimental setting (based on eight media and two temperatures), namely incubation at 55 °C for 5 days on NA CaKMgMn (the composition of the medium is listed in Supplementary Table 1). To obtain the spores, stock cultures (kept at −80 °C) were individually inoculated in 5 mL BHI-B (Brain heart infusion broth) and incubated at 55 °C overnight. Subsequently, 200 μL of overnight culture was spread plated onto plates containing NA CaKMgMn. Sporulated cultures were removed from the surface of the plates after 5 d incubation with cold sterile water, and subsequently washed three times with cold sterile water. Washing was performed by centrifugation of the spore suspensions (5000 × g, 10 min, 4 °C), discarding supernatant, and resuspending of pellets containing the spores. For each strain, two independent spore batches were prepared at independent points in time, using the same experimental conditions. The spore concentrations in the spore suspensions were determined after heating 10 min at 100 °C.

Prior to use in heat inactivation experiments, spore suspensions of all strains were stored at 4 °C for at least one month to allow for spore maturation. The suspensions were assessed using phase contrast microscopy before use, to verify that spores were phase bright (i.e. not germinated). Spore concentrations in the individual batches and the initial counts (N_0) in experiments were determined by heat-treating a spore suspension at 100 °C for 10 min using a PCR machine (GeneAmp PCR System 9700, Applied Biosystems), pour plating 1 mL aliquots of 10-fold serial dilutions (made in sterile water) in TSA, incubation at 55 °C for 5 d, and counting.

2.6. Heat inactivation of spores of 18 different strains

The heat inactivation of spores of the 18 strains was assessed at 125 °C or 130 °C for two individual spore batches per strain, using seven or eight different heating times at each temperature, using the capillary tube method (described above). For two different strains, heat inactivation experiments were repeated five times at 125 °C (spores of strain 4319) and 130 °C (spores of strain 4313) to determine experimental variability. The concentrations of viable spores were determined by pour plating 10-fold serial dilutions (made in sterile water) in TSA followed by incubation at 55 °C for 5 days and enumeration.

2.7. Calculation of D-values and z-values

The $\log_{10}$ of the surviving spores were plotted against the heating time at a given temperature to obtain heat inactivation curves of spores of each spore crop tested. A two-step model fitting method was applied to calculate the *D* and *z*-values. The modified Weibull model (Metselaar et al., 2013) was used to fit each heat inactivation curve because this model is able to fit linear, convex, as well as concave inactivation curves due to function of the shape parameter β.

$$\log N_t = \log N_0 - \Delta\left(\frac{t}{t_{\Delta D}}\right)^{\beta} \tag{1}$$

where $\log N_t$ is the $\log_{10}$ number of the colony count representing the surviving spores at time t in minutes; $\log N_0$is the initial spore concentration ($\log_{10}$ CFU/mL); Δ the reference number of decimal reductions; t is the heat treatment time; $t_{\Delta D}$ the time to reduce the initial spore concentration by Δ decimals, and β is the shape parameter. The average decimal reduction time (D) was calculated by $t_{\Delta D}/\Delta$. The Δ was set at 3 for experiments using spores of strains 9A20 and 4161 that were produced on different media, based on > 3 $\log_{10}$ reductions measured. The Δ was set at 4 for experiments using spores of 18 different strains produced on NA CaKMgMn based on > 4 $\log_{10}$ reductions measured. These Δ values for the two sets of experiments allowed for parameter estimations without extrapolating outside of the experimental range.

The *z*-values were estimated by taking the negative reciprocal of the slope of the line between $\log_{10}$ transformed *D*-values plotted against temperature.

To calculate $\log_{10} D_{\text{ref}}$, Eq. (2) was used

$$\log_{10} D = \log_{10} D_{\text{ref}} + (T_{\text{ref}} - T)/z \tag{2}$$

where $\log_{10} D_{\text{ref}}$ is the $\log_{10}$ of the decimal reduction time (min) at the reference temperature; T_{ref} is the reference temperature; and z is the temperature increase required to reduce the *D*-value by 90%. The fitting procedure was performed in Excel by using Microsoft Solver Add-in.

To calculate the 95% prediction interval of $\log_{10} D_{\text{ref}}$, Eq. (3) was used (Van Asselt and Zwietering, 2006):

$$\log_{10} D_{\text{ref}} \pm t_{DF}\cdot\sqrt{\frac{RSS}{DF}} \tag{3}$$

where t_{DF} is the student *t*-value with degrees of freedom (DF), DF is the number of data (n) used for the linear regression minus the number of parameters, and a significance level of 0.05. RSS is the residual sum of squares calculated from the differences between the linear regression line and $\log_{10}D$-values.

2.8. Quantification of variability of heat resistance of spores of G. stearothermophilus

Experimental variability, reproduction variability, strain variability and history of sporulation variability were quantified in relation to heat inactivation of spores of *G. stearothermophilus*, following an approach similar to the one used in the study by Aryani et al. (2015).

To determine experimental variability (defined as the variability observed for experimental replicates), heat inactivation experiments were repeated five times for two independent spore crops, i.e. for strain 4319 at 125 °C and for strain 4313 at 130 °C. Thus, ten $\log_{10}D$-values were obtained and used to quantify experimental variability through Eq. (4).

$$MSE = \frac{RSS}{DF} = \frac{\sum_{S=1}^{2}\sum_{E=1}^{5}(X_{ES} - X_S)^2}{n - m} \tag{4}$$

where MSE stands for mean square error, X_{ES} is the $\log_{10}D$-value of each experiment E for strain S, X_S is the average $\log_{10}D$-value for strain S, and DF is the number of data points (2 ∗ 5) minus the number of parameters (inactivation temperatures) (2), which is 8.

Reproduction variability, also known as biological variability, is defined as the variability between biologically independent reproductions. For each of 18 strains, two independent spore crops were produced at different points in time and the same two heat treatments (125 °C and 130 °C) were applied for each spore crop. Based on these data sets, reproduction variability was calculated using Eq. (5)

$$MSE = \frac{RSS}{DF} = \frac{\sum_{T=1}^{2}\sum_{S=1}^{18}\sum_{R=1}^{2}(X_{RST} - X_{ST})^2}{n - p} \tag{5}$$

where X_{RST} is the $\log_{10}D$-value of each crop R of strain S at temperature T, X_{ST} is the average $\log_{10}D$-value for strain S at temperature T, and DF is the number of data points minus the number of parameters.

DF = (2 ∗ 18 ∗ 2) − (2 ∗ 18) = 36.

Strain variability is used to describe the variability amongst different strains within the same species. To establish variability in spore heat resistance of *G. stearothermophilus* strains, spores of 18 strains were used in heat inactivation experiments at 125 °C and at 130 °C to characterize the strain variability by using Eq. (6).

$$MSE = \frac{RRS}{DF} = \frac{\sum_{T=1}^{2}\sum_{S=1}^{18}(X_{ST} - X_T)^2}{n-p} \quad (6)$$

where X_{ST} is the average $\log_{10}D$-value of the two crops of strain S at temperature T, X_T is the average of X_{ST} of all strains at temperature T, and DF is the number of data points minus the number of parameters, which is (2 ∗ 18) − 2 = 34.

The impact of history during sporulation, namely the effect of culturing temperature C (55 °C and 61 °C) and agar medium A on variability in spore heat resistance was calculated as follows (Eqs. (7) and (8)), based on spores of two different strains that were produced on six different media at two different temperatures:

$$MSE = \frac{RSS}{DF} = \frac{\sum_{S=1}^{2}\sum_{A=1}^{6}\sum_{C=1}^{2}(X_{CAS} - X_{AS})^2}{n-p} \quad (7)$$

where X_{CAS} is the $\log_{10}D$-value of strain S of which the spores were obtained at culturing temperature C on agar medium A, X_{AS} is the average $\log_{10}D$-value for strain S at agar medium A, and DF is the number of data points minus the number of parameters. DF = (2 ∗ 6 ∗ 2) − (2 ∗ 6) = 12.

$$MSE = \frac{RSS}{DF} = \frac{\sum_{S=1}^{2}\sum_{C=1}^{2}\sum_{A=1}^{6}(X_{CAS} - X_{CS})^2}{n-p} \quad (8)$$

where X_{CAS} is the $\log_{10}D$-value of strain S of which the spores were obtained at culturing temperature C on agar medium A, X_{CS} is the average $\log_{10}D$-value for strain S at culturing temperature C, and DF is the number of data points minus the number of parameters. DF = (2 ∗ 6 ∗ 2) − (2 ∗ 2) = 20.

Also the combined effect of culturing temperature and agar medium on spore heat resistance was determined:

$$MSE = \frac{RSS}{DF} = \frac{\sum_{S=1}^{2}\sum_{C=1}^{2}\sum_{A=1}^{6}(X_{CAS} - X_{S})^2}{n-p} \quad (9)$$

where X_{CAS} is the $\log_{10}D$-value of strain S of which the spores were obtained at culturing temperature C on agar medium A, X_S is the average $\log_{10}D$-value for strain S, and DF is the number of data points minus the number of parameters. DF = (2 ∗ 6 ∗ 2) − 2 = 22.

To compare different variabilities, the F-test was used:

$$F = \frac{MSE_1}{MSE_2} \quad (10)$$

where MSE_1 is the mean square error of the first variability factor, MSE_2 is the mean square error of the second variability factor, and a significance level of 0.05.

2.9. Meta-analysis on spore inactivation of *G. stearothermophilus*

In food microbiology, meta-analyses are increasingly adopted to evaluate growth or resistance parameters of a certain microorganism of concern in food (Halder et al., 2010). To compare and combine results from the current study with data available in literature on heat resistance of *G. stearothermophilus* spores, a meta-analysis was performed by collecting *D*-values available in literature. This meta-analysis was built on the one published by Rigaux et al. (2013) which was based on 430 *D*-values as previously published (Anderson and Friesen, 1974; André et al., 2009, 2012; André, 2011; Cook and Gilbert, 1968a, 1968b; Couvert, 2002; Davies et al., 1977; Etoa and Michiels, 1988; Feeherry et al., 1987; Fernandez et al., 1994, 1995; Gauthier et al., 1978; Haas et al., 1996; Le Jean et al., 1994; López et al., 1996a, 1996b, 1997, 1998; Mallidis and Scholefield, 1986; Ocio et al., 1996; Patazca et al., 2006; Periago et al., 1998a, 1998b, 1998c; Rodrigo et al., 1997, 1999; Tejedor et al., 2001; Wallace et al., 1978; Wescott et al., 1995). For the current meta-analysis, additional *D*-values published in some of the above mentioned studies but not included in the meta-analysis by Rigaux et al. (2013) were included as well (Haas et al., 1996; Fernandez et al., 1995; López et al., 1996a, 1998; Ocio et al., 1996; Periago et al., 1998b; Rodrigo et al., 1999). In addition, *D*-values from additional studies were included (André et al., 2013; Bender and Marquis, 1985; Dlugokenski et al., 2011; Durand et al., 2015; Huemer et al., 1998; Leontidis et al., 1999; Somavat et al., 2012; Yildiz and Westhoff, 1989), rendering a total of 649 $\log_{10}$-transformed *D*-values extracted from scientific publications. Regarding data collection, if *D*-values or $\log_{10}D$-values were reported in the literature, these were taken as such and *D*-values were $\log_{10}$-transformed; if only inactivation curves or 4*D*-values were published, then *D*-values were calculated manually.

3. Results

3.1. Enumeration of *G. stearothermophilus* spores

Eight different media which were described in literature (overview given in Supplementary Table 1) were evaluated for the ability to support germination and outgrowth of heat-treated spores of strains 9A20 and 4161. Spores of strain 9A20 and 4161 (produced on NA CaKMgMn at 55 °C for 5 days) that were heated for 10 min 100 °C showed recoveries of around 8 $\log_{10}$ CFU/mL on AAMS, PCMA ISO, NA, SA, TSA and TSA Ca (Fig. 1). However, for both strains, spore counts were approximately 2 $\log_{10}$ units lower on TBAB and for strain 4161, spore counts were also approximately 2 $\log_{10}$ units lower on BHI-A B12, indicating that these two media were less suitable for recovery of spores.

Subsequently, the eight cultivation media were used to evaluate growth of six additional strains of *G. stearothermophilus* (4109, 4111, 4112, 4114, 4163 and 9A2). Growth of vegetative cells of these strains was supported on AAMS, PCMA ISO, NA, SA, TSA and TSA Ca (Table 2). Of these media, PCMA ISO and TSA are commonly used in practice. While the counts were the same on these two media, the colonies on TSA were larger and easier to count than on PCMA (rendering small pinpoints). Medium BHI-A B12 supported growth of five strains, but not of strain 4114. Medium TBAB did not support growth of strains 4111, 4114, 4163 and 9A2 and only limited growth was observed for strains 4109 and 4112. Results obtained after 3 and 6 days of incubation were identical.

Overall, the recovery of spores as assessed by the appearance of colonies on agar media depended on the strain used and the media on which spores were plated. BHI-A B12 and TBAB did not consistently support good recovery of spores. Whereas NA supported growth to

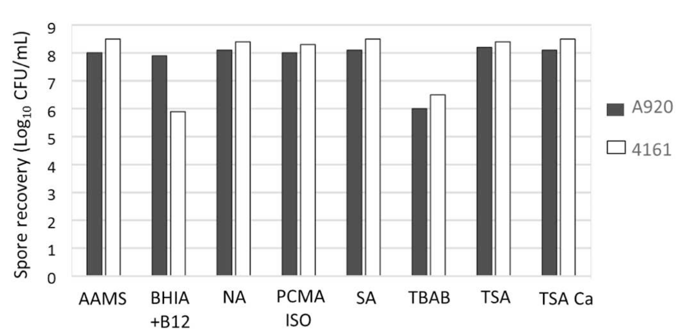

Fig. 1. Spore recovery of *G. stearothermophilus* strain 9A20 and 4161 on different media. Data represent the mean of duplicate plate counts after 5 days incubation at 55 °C. Spores used (obtained by sporulation on NA CaKMgMn at 55 °C) were subjected to heating for 10 min 100 °C prior to plating.

Table 2
Assessment of the ability of vegetative cells of six different *Geobacillus stearothermophilus* strains to grow on different media after streaking (incubation at 55 °C for maximally 6 days). Visibility of colonies was assessed (+ = growth, − = no growth, * = 1 to 3 individual colonies).

Medium	Strain					
	4109	4111	4112	4114	4163	9A2
AAMS	+	+	+	+	+	+
BHI-A B12	+	+	+	−	+	+
NA	+	+	+	+	+	+
PCMA ISO	+	+	+	+	+	+
SA	+	+	+	+	+	+
TBAB	+*	−	+*	−	−	−
TSA	+	+	+	+	+	+
TSA Ca	+	+	+	+	+	+

visual colonies, recoveries of heat-damaged spores was sub-optimal (Supplementary Fig. 1). For enumeration purposes, plating on BHI-A B12, TBAB and NA may thus lead to an underestimation of viable counts of *G. stearothermophilus*. TSA showed the most robust colony formation and good recovery.

3.2. Spore yields using different media

Spore production of strains 9A20 and 4161 was assessed at 55 °C and 61 °C using various sporulation media, including two milk-based agars and in a milk-biofilm system. After harvesting the lawns, spore yields ranged from 8.7 $\log_{10}$ CFU/mL to < 4 $\log_{10}$ CFU/mL (Table 3). For both strains tested, spore yields varied per method, with the highest yields obtained on NA CaMn and NA CaKMgMn. No significant differences in yields were seen when culturing at 55 or 61 °C, with the exception of strain 4161 on TSA, showing nearly 2 $\log_{10}$ lower spore yields at 61 °C than at 55 °C (Table 3). PCA5M, milk and the milk biofilm system (liquid and metal coupon surface) gave relatively low spore counts (maximally 5 $\log_{10}$ CFU/mL). No bacterial lawn was formed on any of the milk agar plates.

Table 3
Yields of spores of *Geobacillus stearothermophilus* strains 9A20 and 4161 produced at 55 °C or 61 °C on various solid media and in a milk-biofilm system (from liquid phase and on surface coupons). Yields were determined by plating on TSA after heating (100 °C for 10 min). Data are expressed in $\log_{10}$ CFU/mL and represent the mean of duplicate plate counts. The time needed for 1 $\log_{10}$ reduction of the spores at 125 °C ($D_{125°C}$) was determined based on (t_{3D}/3) at 125 °C and is presented as well.

		Strain 9A20		Strain 4161	
Sporulation medium	Sporulation temp (°C)	Spore yield ($\log_{10}$ CFU/mL)	$D_{125°C}$ (min)	Spore yield ($\log_{10}$ CFU/mL)	$D_{125°C}$ (min)
NA CaKMgMn	55	8.2	1.83	8.6	0.74
	61	8.1	2.00	8.6	0.52
NA CaMn	55	8.7	1.14	8.0	0.51
	61	7.8	1.63	7.5	0.45
TSA	55	7.2	1.50	7.0	0.46
	61	7.2	1.05	5.4	0.51
TSA CaKMgMn	55	7.3	1.89	8.1	0.58
	61	7.5	2.13	8.2	0.56
YBP	55	7.5	1.26	8.1	0.53
	61	7.3	1.47	8.4	0.54
YMMn	55	7.6	0.66	6.9	0.063
	61	7.8	0.64	6.2	0.093
Milk agar	55	–*	ND	–*	ND
	61	–*	ND	–*	ND
PCA5M	55	4.8	ND	< 4.0*	ND
	61	5.0	ND	< 4.0*	ND
Milk from biofilm system	55	< 4.0*	ND	5.0	ND
	61	4.9	ND	4.0	ND
Metal coupon swab (biofilm)	55	< 4.0*	ND	4.3	ND
	61	< 4.0*	ND	< 4.0*	ND

– = no bacterial lawn formed and no spores harvested, ND = not determined.
* detection limit.

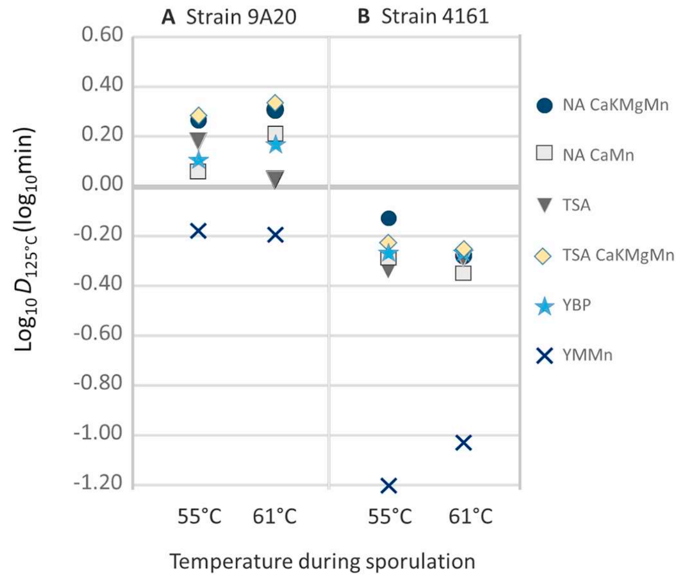

Fig. 2. Heat inactivation of spores of *Geobacillus stearothermophilus* strains 9A20 (A) and 4161 (B). Data presented show the logarithm of the average time needed for 1 $\log_{10}$ reduction at 125 °C ($\log_{10}D_{125°C}$ values, based on t_{3D}/3). Spores were produced on NA CaKMgMn, NA CaMn, TSA, TSA CaKMgMn, YBP, and YMMn at 55 °C and 61 °C.

Spores produced on NA CaKMgMn, NA CaMn, TSA, TSA CaKMgMn, YBP and YMMn were used in subsequent experiments to establish heat resistance of spores. Microscopic analysis of the washed spore batches revealed the presence of vegetative cells in all spore suspensions (which were inactivated by heating for 10 min at 100 °C).

3.3. Influence of sporulation conditions on spore heat resistance

The heat resistances of spores produced on six different media (NA CaKMgMn, NA CaMn, TSA, TSA CaKMgMn, YBP and YMMn) at two incubation temperatures (55 °C and 61 °C) of two different strains (9A20 and 4161) are presented in Table 3 (D_{125}-values) and in Fig. 2 ($\log_{10}D_{125}$-values) as the mean of the two independent experiments per strain per temperature. For many data sets the curve parameter β was significantly different from 1 (data not shown), advocating the use of the Weibull model instead of the log-linear model.

In line with the observations presented in Section 3.1, spores of strain 9A20 were generally more heat resistant than spores of strain 4161. For both strains, spores produced on NA or TSA supplemented with CaKMgMn showed the highest heat resistance while spores produced on NA CaMn, TSA or YBP and YMMn were less resistant.

Spores of strain 9A20, sporulated on NA CaKMgMn and TSA CaKMgMn at 61 °C, showed the highest heat resistance with D_{125}-values of 2.13 and 2.00 min, respectively (Table 3). Spores of strain 4161 showed the highest heat resistance when produced on NA CaKMgMn and TSA CaKMgMn at 55 °C compared with other conditions, with D_{125}-values of 0.74 and 0.58 min, respectively (Table 3). Thus, for spores produced under conditions favouring high level heat resistance, spores of strain 9A20 required approximately three times longer heating times at 125 °C than those of strain 4161 to achieve one decimal reduction, reflecting the impact of strain type on spore heat resistance. The impact of sporulation medium on spore heat resistance was also high: on YMMn, spores of strain 9A20 showed D_{125}-values of only 40 s and 38 s for spores produced at 55 and 61 °C, respectively. For strain 4161 the D_{125}-values were even lower (only 3.8 and 5.6 s for spores produced at 55 and 61 °C, respectively).

The variability in spore heat resistance due to history of sporulation (*H*) for the two different strains based on all sporulation conditions tested (2 temperatures, 6 media) expressed in RMSE was 0.27. The variability could largely be attributed to the use of different agar media (*A*; RMSE 0.28) while the variability in spore heat resistance due to culturing temperature of 55 or 61 °C during sporulation was much lower (*C*; RMSE 0.07).

3.4. Heat resistance of spores of 18 strains of G. stearothermophilus

Sporulation conditions rendering high yields of spores with high level heat resistance as established for strains 9A20 and 4161 (i.e. NA CaKMgMn at 55 °C for 5 d), were used to produce and harvest spores of a total of 18 strains of *G. stearothermophilus* in duplicate, i.e. two independent spore crops per strain. For this large scale spore production, incubation at 55 °C was chosen, as the stability of agar at 61 °C was impaired. Time-temperature combinations for heating were applied that rendered at least 4 $\log_{10}$ reductions of spores (compared with initial spore levels N_0, determined by heating for 10 min 100 °C), from which the average *D*-values at different temperatures were derived upon fitting using the modified Weibull model. The average $\log_{10}D_{125}$ and $\log_{10}D_{130}$-values of all 18 strains are presented in Fig. 3. The actual *D*-values per spore batch per strain at 125 °C and 130 °C and the average values per strain at the two temperatures are presented in Supplementary Table 3. The plots of actual inactivation data at both temperatures are presented in Supplementary Fig. 2.

Overall, strains 9A20 and 4112 produced spores with the highest heat resistance while spores of strains 4310 and 4161 were the most heat sensitive. The average D_{125}-values showed a factor 4.4 difference and ranged from 0.53 min for the least heat resistant spores of strain 4310 to 2.34 min for the most heat resistant spores (of strain 9A20), respectively (Supplementary Table 3). For the latter strain, D_{125}-values of 1.83 and 1.89 min were seen for spores produced at 55 °C on Na CaKMgMn and TSA CaKMgMn in the initial tests (Table 3), which were in line with these findings. Using a heating temperature of 130 °C, the average D_{130}-values differed a factor 4 and ranged from 0.20 min for the least heat resistant spores (again strain 4310) to 0.77 min for the most heat resistant spores (of strain 4112), respectively. Spores of strain 4161 showed the second lowest heat resistance, with a D_{125}-value of 0.58 min and D_{130}-value of 0.28 min (Fig. 3, Supplementary Table 3).

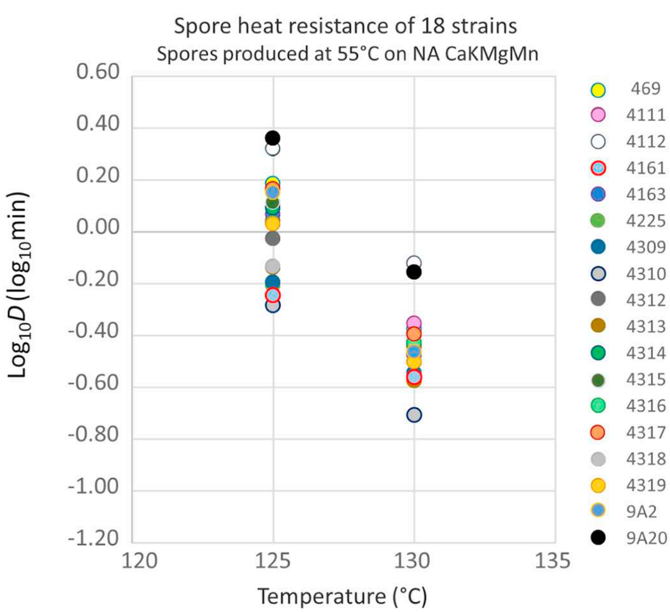

Fig. 3. Heat inactivation of spores of 18 different *Geobacillus stearothermophilus* strains. Data presented show the logarithm of the average time needed for 1 log reduction at 125 °C or at 130 °C ($\log_{10}D$-values based on $t_{4D}/4$). All spores were produced on NA CaKMgMn at 55 °C.

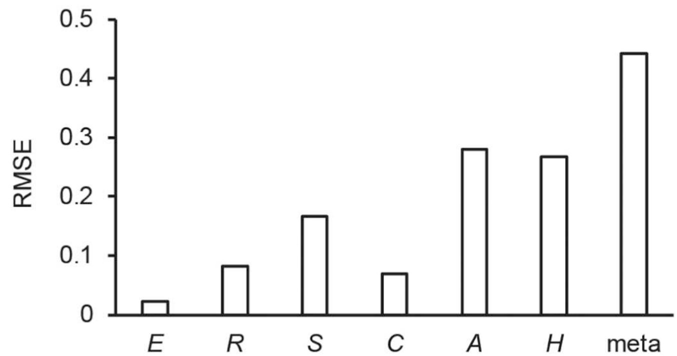

Fig. 4. Experimental (*E*), reproduction (*R*), strain (*S*), culturing temperature (*C*), agar medium (*A*), history of sporulation (*H*), and meta analysis variability of heat resistance spores of *Geobacillus stearothermophilus*, expressed in the standard deviation RMSE.

In Fig. 4, the impact of experimental procedures, the use of different strains and culturing conditions on variability in spore heat resistance is presented. Based on the data sets of the spore inactivation presented in Supplementary Table 3 and the inactivation of spores of two separate spore crops (one of strain 4319 and one of 4313) as repeated five times ($\log_{10}D$-values presented in Supplementary Table 4), it was established that strain variability (*S*; RMSE 0.17) was two times larger than reproduction variability (*R*; RMSE 0.08) and 7.5 times larger than experimental variability (*E*; RMSE 0.02). The variability due to history of sporulation (*H;* RMSE 0.27) was the highest, followed by strain variability (*S*; RMSE 0.17). The variability due to history of sporulation H could largely be attributed to the use of different agar media as mentioned for spores produced at 55 and 61 °C.

3.5. Comparing heat inactivation data from the current study with data from literature

For the meta-analysis, a total of 649 log10-transformed *D*-values were extracted from scientific publications. The $\log_{10}D$-values were obtained from heat inactivation experiments of different *G. stearothermophilus* (formerly *Bacillus stearothermophilus*) strains from different media, at temperatures ranging from 85.6 °C to 140 °C and pH values ranging from 3 to 7. An overview of the data is presented in Fig. 5A, in which all $\log_{10}D$-values are plotted against temperature. The 95% prediction intervals were calculated based on the data collected from the literature. The variability expressed in RMSE for the data of the meta-analysis (M) was 0.44 (Fig. 4). In Fig. 5B, the $\log_{10}D$-values of spores of strains 9A20 and 4161 that were produced on six different media at 55 °C and 61 °C are plotted against temperature within the 95% prediction interval of the meta-analysis. This clearly highlights the importance of sporulation conditions on spore heat resistance, with variability of sporulation history contributing to approximately half of the overall variability as seen in the meta-analysis. In Fig. 5C, the average $\log_{10}D$-values at 125 °C and 130 °C of spores of the 18 strains, produced under conditions rendering spores with the highest heat resistances, are plotted against temperature within the 95% prediction interval of the meta-analysis. The $\log_{10}D$-values obtained in the current study were somewhat at the higher end of the 95% prediction interval of all literature data. The strains variability of spores produced under the same conditions contributes to nearly half of the overall variability as seen in the literature.

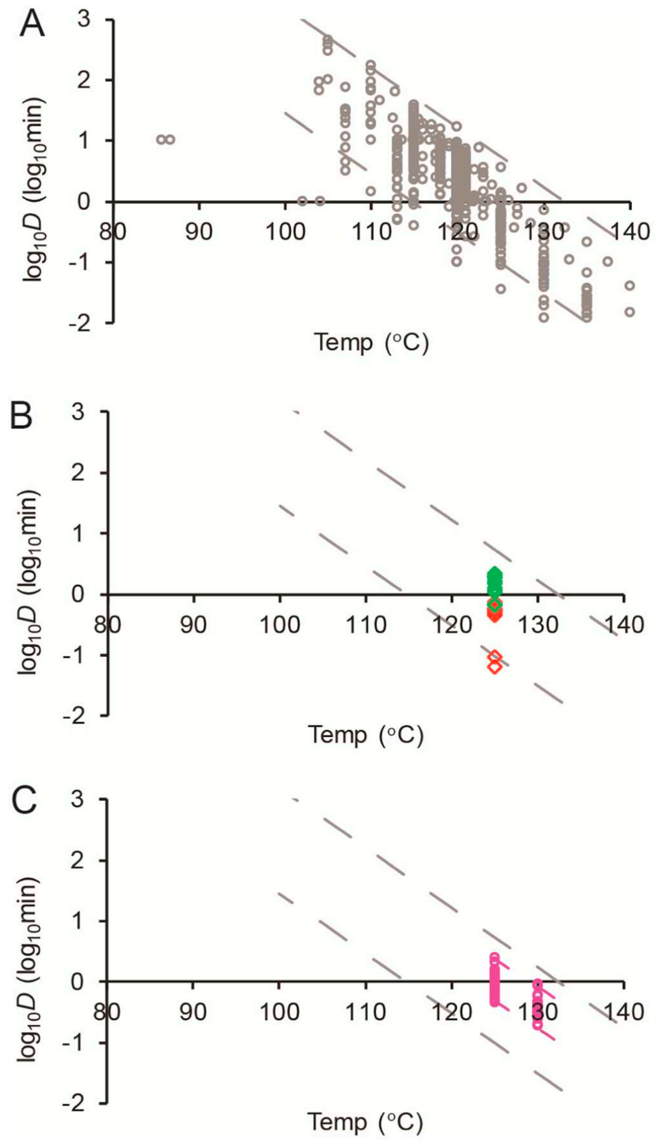

Fig. 5. (A) Meta-analysis of heat resistance of spores of *Geobacillus stearothermophilus* showing $\log_{10}$ *D*-values of *G. stearothermophilus* spores as extracted from the scientific literature plotted against given temperatures; (B) $\log_{10}$ *D*-values of spores of strains 9A20 (green dots) and 4161 (red dots) produced at 55 or 61 °C using six different media; (C) $\log_{10}$ *D*-values of spores of 18 different strains that were produced on NA CaKMgMn at 55 °C (purple dots). The dashed lines represent the 95% prediction intervals. Outliers fall outside the 95% prediction interval. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Overall, data obtained from literature and generated in the current study showed a high variability in heat resistance of spores of *G. stearothermophilus*, with sporulation history (depending mostly on the sporulation medium at 55 and 61 °C) and strain variability being the most important factors.

4. Discussion

The thermophilic bacterium *G. stearothermophilus* can lead to commercial non-sterility in low acid foods when spores survive sterilization processes, followed by germination and growth at elevated storage temperatures (generally above 40 °C). Factors investigated in this study included suitability of cultivation media for enumeration of *G. stearothermophilus* spores, the variation in heat resistance of spores produced under different conditions, and the variability in spore heat resistance based on a broad range of strains of *G. stearothermophilus*, mostly isolated from foods.

4.1. Enumeration of *G. stearothermophilus* spores

In the current study, eight media described in literature for the cultivation of *G. stearothermophilus* were evaluated for the recovery of spores. Spores were first heat treated for 10 min at 100 °C. This heat shock was performed to inactivate vegetative cells, and in addition, it has been reported that recovery of spores of *G. stearothermophilus* spores is somewhat better after such heat treatment compared with no heating prior to plating. As mentioned above, Cook and Gilbert (1968a) reported 1.3 times higher recoveries of *G. stearothermophilus* spores upon heat treatments of 10 min at 115 °C compared with unheated spores. Finley and Fields (1962) also reported activation of spore germination at temperatures above 100 °C, e.g. 1.4 times higher counts after heating for 15 min at 105 °C and 1.9 times higher counts after heating for 7 to 10 min at 110 °C, compared with the unheated control. Feeherry et al. (1987) also showed up to 1.9 times higher recoveries of spores after heating for 15 min 100 °C compared with unheated spores. While these studies all show maximally 2-fold increased recoveries of spores, it should be noted that on a $\log_{10}$ scale this is maximally 0.3 $\log_{10}$ units, and such differences may not be significant when establishing heat inactivation experiments, with starting concentrations of ~8 $\log_{10}$ spores/mL and reductions exceeding 3 $\log_{10}$ log units.

A number of cultivation media appear to be unsuitable for recovery of heat treated spores of *G. stearothermophilus*. This included TBAB, which is recommended by the BGSC as the best general purpose solid medium for *Geobacillus* spp. (Zeigler, 2001), and BHI-A B12, a commonly used standard medium for cultivation of *Bacillus* species. NA supported full recovery of spores of strains 9A20 and 4161 after heat treatment of 10 min 100 °C, however, recovery of heat-damaged spores of strain 4161 after heating for 90 s at 125 °C was lower on this medium than on AAMS, PCMA ISO, SA, TSA, and TSA Ca (Supplementary Fig. 1).

Overall, TSA was considered to be the best recovery medium, yielding clear, easily countable colonies, supporting germination and outgrowth of the tested strains. The addition of calcium to TSA, which has previously been reported to increase the recovery of *G. stearothermophilus* spores (Dlugokenski et al., 2011), seems to be unnecessary considering similar recoveries of spores on TSA and TSA Ca. The addition of starch to cultivation media as an aid to spore germination has a long standing history (Cook and Gilbert, 1968a; López et al., 1997; Mallidis and Scholefield, 1986) and is common practice when determining thermophilic spore counts (Burgess et al., 2010; ISO/TS 27265:2009). The media that contained starch in the present study (AAMS, SA and PCMA ISO) did not yield higher counts than non-starch media such as TSA or TSA Ca. Dlugokenski et al. (2011) also reported that the presence of soluble starch to cultivation medium did not result in a greater germination and outgrowth capacity for heat treated spores of *G. stearothermophilus*. In view of the results presented in this study and other similar findings in our laboratory (Eijlander and Wells-Bennik, unpublished), the addition of starch also appears to be unnecessary for recovery of *G. stearothermophilus* spores.

4.2. Variation in spore heat resistance

The heat resistances of spores of *Geobacillus stearothermophilus* have been determined in many studies. Rigaux et al. (2013) published a meta-analysis on the heat inactivation of *G. stearothermophilus* spores, based on a set of 430 *D*-values reported in literature. These authors reported a mean $D_{121.1}$-value of 3.3 min with their analysis. The 95% prediction interval for $D_{121.1}$ ranged from 0.8 to 9.6 min (95% PI = (0.8; 9.6)), and the authors calculated a mean *z*-value of 9.1 °C (SD = 1.9 °C). This illustrates that considerable variation exists in the heat resistance of spores of *G. stearothermophilus*. Spore inactivation data available in literature are based on experiments carried out with different strains, in different food matrices, using different spore batches produced on different media, and using various recovery media. The meta-analysis presented in the current study included 649 data points, and it was built on the data set of Rigaux et al. (2013). This rendered in our analysis a mean $\log_{10}D_{121.1}$-value of 0.24 (95% PI $\log_{10}D_{121.1} = -0.63$; 1.11), equaling a mean $D_{121.1}$ of 1.7 min (95% PI = (0.23; 12.9 min)). The calculated z-value based on the new meta-analysis is 10.2 min. Based on the $\log_{10}$ D-values of spores of all 18 strains determined at 125 °C and 130 °C in duplicate, a z-value of 11.1 °C was calculated. Although the latter value is based on measurements (18 strains reproduced) at only two different temperatures (i.e. 72 data points), it is in line with the z-value based on the meta-analysis (based on 649 data points).

Generally, positive correlations have been established between the maximum growth temperatures of *Bacillus* species and the heat resistances of their spores (Michels and Visser, 1976; Warth, 1978) although significant variation may exist between strains of species (Berendsen et al., 2016a, 2016b; Butler 3rd et al., 2017; Den Besten et al., 2017). In addition, positive correlations have been reported between the temperature of sporulation and the thermal resistance of the spores (Baril et al., 2011, 2012; Cook and Gilbert, 1968b; Huo et al., 2012; Leguérinel et al., 2007) and it is known that sporulation conditions can have an impact on the heat resistance of spores (Cook and Gilbert, 1968a, 1968b; Guizelini et al., 2012; Leguérinel et al., 2007; López et al., 1997; Williams and Robertson, 1954). However, no systematic evaluation of the impact of these variables on spore heat resistance of *G. stearothermophilus* was available so far.

In this study, spores of the 18 *G. stearothermophilus* strains were produced under the same conditions, rendering spores with high level heat resistances. At the reference temperature of 121.1 °C, the mean $\log_{10}D_{121.1}$ was 0.37 (95% PI $\log_{10}D_{121.1} = 0.03$; 0.71), equalling a mean $D_{121.1}$ of 2.4 min (95% PI $D_{121.1}$ = (1.1 min; 5.2 min)). These data indicate that strain variability contributes significantly to overall variability in spore heat resistance of *G. stearothermophilus*, accounting for approximately 40% of the total variation seen in the meta-analysis. Genetic differences clearly exist between different strains of *G. stearothermophilus* as demonstrated by Durand et al. (2015). Differences in genes that encode proteins involved in sporulation may underlie such differences in spore heat resistance, as has been demonstrated for *Bacillus* and *Clostridium* species (Berendsen et al., 2016a, 2016b; Butler 3rd et al., 2017). Whether this is the case for *Geobacillus* species remains to be elucidated.

Additionally, conditions during sporulation had a significant effect on spore heat resistance, contributing to around half of the overall variability in spore heat resistance as seen in the meta-analysis. It was found that spores of the two tested strains were clearly more heat resistant when produced on NA or TSA supplemented with CaKMgMn than on other sporulation media, indicating an essential role of free ions like Ca^{2+} K^+ Mg^{2+} and Mn^{2+} ions in the acquisition of heat resistance of *G. stearothermophilus* spores. A number of other tested sporulation media, such as NA CaMn, YBP and YMMn, also contained some of these ions, albeit in different concentrations and/or combination. The heat resistances of spores produced on those media were generally lower. This highlights the importance of Ca^{2+} K^+ Mg^{2+} and Mn^{2+} in sporulation agar medium for the production of heat resistant spores, similar to what has been reported for *Bacillis subtilis* (Cazemier et al., 2001; Oomes and Brul, 2004; Schaeffer et al., 1965). Surprisingly, spores produced on YMMn, which contained the divalent cation Mn^{2+}, were shown to be the most heat sensitive in the present study with a D_{125}-value of 0.66 min, which appeared to be in contrast with the study by Guizelini et al. (2012) who reported this formulation to induce maximal heat resistance in *G. stearothermophilus* spores. However, when comparing the heat resistance of spores of strain 9A20 as produced on YMMn with data from literature, similar heat resistances were found for spores of this strain that were produced on a medium which was highly similar to YMMn (10 g/L meat extract, 2 g/L yeast extract, 0.04 g/L $MnSO_4 * H_2O$ and 15 g/L agar) (Durand et al., 2015). At a temperature of 120 °C, Durand et al. (2015) reported a t_{5D}-value (time for a 5-$\log_{10}$ reductions) of 12.4 min (equals average D_{120}-value of 2.5 min) for spores of this strain produced on the latter medium. Assuming a z-value of 10 °C, the reported t_{5D}-value can be converted to a D_{125}-value of 0.79 min, which is close to the D_{125}-value of 0.66 min.

In the current study, the differences in spore heat resistance as affected by sporulation at 55 or 61 °C were small. For *G. stearothermophilus* specifically, different heat resistances of spores of different strains of *G. stearothermophilus* in response to sporulation temperature have been reported previously (Williams and Robertson, 1954). These authors cultured five strains at 37 °C, 45 °C and 55 °C and five at 55 °C and 60 °C and showed that spores produced at 55 °C had 1.4 to 3.5 longer survival times at 110 °C than those produced at 37 °C, while spores produced at 60 °C showed 1.1 to 1.5 times longer survival times at 120 °C than those produced at 55 °C. In addition, Cook and Gilbert (1968b) reported increases in the decimal reduction times from 12.2 to 16.2 to 24.4 min at 115 °C for spores of *G. stearothermophilus* produced at 50, 55, or 60 °C, respectively. In the present study, spores produced at 61 °C were not always more heat resistant than those produced at 55 °C, showing no conclusive effect. Larger effects of sporulation temperature on spore heat resistance of *G. stearothermophilus* may be seen when spores are produced at temperatures further away from the optimal growth temperatures (for instance at 40 or 50 °C).

Taken together, the variability in spore heat resistance as established in this study is mostly due to strain variability and cultivation medium conditions during spore formation. The variability due to these two factors were much larger than the experimental and reproduction variability and the variability as a result of sporulation at 55 or 61 °C. The selected combinations of different strains and sporulation conditions covered most of the overall variation observed in the meta-analysis (Fig. 5). Spores with relatively low heat resistances (low end of the 95% prediction interval) could be obtained using a strain producing heat sensitive spores (4161) when sporulated on medium that did not support the production of heat resistant spores (YMMn). On the other hand, spores with heat resistances at the high end of the 95% prediction interval were produced using a strain (9A20) producing the most heat resistant spores when sporulated on medium that supported the production of heat resistant spores (NA CaKMgMn).

A factor that may contribute to further variation in measured spore heat resistances is the heating menstruum, which was not in the scope of this study. Negative effects on spore recovery and heat resistance of *G. stearothermophilus* spores have been reported for phosphate (Cook and Gilbert, 1968b; Finley and Fields, 1962; Gauthier et al., 1978; López et al., 1996b, 1997) and acidification (Bender and Marquis, 1985; Fernandez et al., 1994) of the heating menstruum. Another example of the impact of heating menstruum was given in the study by Fernandez et al. (1994), who reported lower D-values of *G. stearothermophilus* obtained from mushroom extract than from bi-distilled water. Clearly, the effect of the heating menstruum on spore heat inactivation is relevant to foods. When this effect is better understood, it could be taken into consideration in validation studies or in modelling of inactivation of spores in foods.

4.3. Implications for production of heat resistant spores for sterilization tests

When validating the effect of certain heat treatments on inactivation of spores in actual products, clearly, it is important to use spores with appropriate heat resistance properties, as the use of spores with insufficiently high heat resistances will lead to an overestimation of the effectiveness of the sterilization process. Spores of strains 9A20 and 4112 showed the highest heat resistance. Strain 9A20 (= ATCC 12980^T) is used as a biological indicator for steam sterilization (Anonymous, 1995; Periago et al., 1998b). Strain 4112 (= T26) was isolated from pea

Table 4
Mean reduction of spores of *G. stearothermophilus* ($\log_{10}$CFU/mL) and corresponding minimal reduction (based on 95% PIs of $\log_{10}D$-value) according to heat treatment regime indicated.

Applied heat treatment	Heat treatment regime	Log_{10} reduction of spores based on extended meta-analysis ($\log_{10}$ CFU/mL) Mean (minimum)	Log_{10} reduction of spores based on data obtained with 18 strains ($\log_{10}$ CFU/mL) Mean (minimum)
Minimum '*Clostridium botulinum* cook' $F_0 = 3$	3 min 121 °C	1.7 (0.2)	1.3 (0.6)
Safety margin on botulinum cook ($F_0 = 5$)	5 min 121 °C	2.8 (0.4)	2.1 (1.0)
Ultra high temperature (UHT)	4 s 135 °C	0.90 (0.12)	0.50 (0.23)
	4 s 140 °C	2.8 (0.4)	1.4 (0.6)
	4 s 145 °C	8.7 (1.2)	4.0 (1.8)
Heat treatment prior to enumeration of highly heat resistant spores	30 min 100 °C	0.14 (0.02)	0.16 (0.07)
	30 min 106 °C	0.56 (0.08)	0.56 (0.26)

soup (Oomes et al., 2007).

The wet heat resistance of spores of strain 9A20 has been the subject of investigation in several other studies. Out of the 18 strains tested in this study, the highest D_{125}-values were found for spores of this strain (2.16 and 2.53 min for crop 1 and 2, respectively) (Supplementary Table 3), corresponding with D_{115} of 21.6 and 25.3 min when assuming a z-value of 10. These values are very similar to previously reported heat inactivation data on spores of this strain as measured in water, namely, a D_{115} of 23.3 min (Fernandez et al., 1994) and a D_{115} of 28.6 min (Periago et al., 1998b). For spores of *G. stearothermophilus* strain NCIB 8919, the highest reported D_{115}-value was even a bit higher for spores heated in distilled water, namely 30 min (as calculated from $t_{4D}/4$) (Cook and Gilbert, 1968a).

The heat resistances of spores of strain 9A20 (ATCC 12980^{T}) as reported in literature were considerably lower than the ones presented above when additional stresses were applied during heating, such as decreased pH or added NaCl. For instance, López et al. (1996a) reported D_{125}-values between 0.13 and 0.62 min for spores suspended in McIlvaine buffer at pH values ranging from 4 to 7; Periago et al. (1998b) obtained D_{125}-values ranging from 0.06–0.62 min for spores heated in mushroom extract using different combinations of pH (5.75–6.5) and NaCl concentrations (0.5–3%); and Rodrigo et al. (1997) reported a mean D_{125}-value of 1.5 min when spores were heat treated in a solution containing 2% $CaCl_2$ (which was expected to boost resistance).

For spores of strain 4161, the highest D_{125}-value found was 0.74 min upon spore production on NA CaKMgMn (Table 3). The spore heat resistance of strain 4161 has also been examined by Zhao et al. (2013) who determined a D_{110} of 18 min, which can be converted to a $D_{125^\circ C}$ of 0.78 min using the reported z-value of 11 °C. Although Zhao et al. (2013) produced their spores on NA CaMn (giving $D_{125^\circ C}$ 0.53 min in this study, see Table 3), the heat resistance of these spores is highly comparable to the highest estimate obtained in the present study.

Other strains that are reportedly used for sterilization tests include strains 9A2 (= NRRL B4419) (Zeigler, 2001) and 4314 (= DSMZ 5934; ATCC7953) (Sasaki et al., 2000). Amongst the 18 strains tested, these strains produced spores that were less heat resistant than those of strain 9A20. Their spores showed intermediate heat resistances, as did other tested strains that were isolated from pea soup or canned peas, such as strain 4111 and 4313, and strains isolated from sugar beet juice, such as strain 4309 and 4310.

It can be concluded that the relatively high D-values found for strain 9A20 are comparable with some reported values in other studies. To obtain such heat resistances, care needs to be taken with the selection of sporulation conditions to the formation of high level heat resistant spores, the heating menstruum of spores, and plating on optimal recovery medium at 55 °C. When high level heat resistant spores of *G. stearothermophilus* are required for sterilization tests, the use of strain 9A20 is recommended, using sporulation medium NA CaKMgMn and incubation at 55 °C.

4.4. Implications for heat treatments applied in practice

The effects on inactivation of spores of *G. stearothermophilus* of typical sterilization and UHT heat treatments that are applied regularly in the food industry are presented in Table 4. The estimations of the mean and minimum inactivation of spores of *G. stearothermophilus* are given, based on the calculated D- and z-values on the basis of the meta analysis (with a relatively broad distribution) and the data sets on 18 strains (with a more narrow distribution; all spores were produced under conditions that favoured production of heat resistant spores).

When sterilization processes are applied at 121 °C, a 3 min heating time is expected to result in a mean reduction of 1.7 $\log_{10}$ units for *G. stearothermophilus* spores, but for the more resistant spores (with upper 95% PI $\log_{10}D$-value) only a 0.2 $\log_{10}$ unit reduction is expected. When increasing the time to 5 min, the mean reduction increases to 2.8 $\log_{10}$ with a minimum of 0.4 $\log_{10}$ units (Table 4). UHT treatments of 4 s at 135, 140 or 145 °C are estimated to result in mean reductions of 0.9, 2.8, or 8.7 $\log_{10}$ units, respectively. However, for the most heat resistant spores of *G. stearothermophilus*, time temperature combinations of > 4 s at 140 °C are needed to inactivate the spores by > 1 $\log_{10}$ unit (Table 4). However, as higher heating conditions may be detrimental to product quality, this may be undesirable in practice. Therefore, there is a need for low concentrations of such spores in ingredients used to produce commercially sterile foods that may be exposed to high temperatures in the distribution chain. In addition, the effect of common heat treatments that are applied for the enumeration of high level heat resistant spores was assessed. When applying heat for 30 min at 100 °C, only limited inactivation of spores of *G. stearothermophilus* is expected (< 0.2 $\log_{10}$) based on calculated D-values. When 30 min 106 °C is applied, a mean reduction of around 0.6 $\log_{10}$ reductions is expected (with 0.3 $\log_{10}$ for the most heat resistant spores).

When calculating heat inactivation of spores, it should be noted that D-values are generally based on the average time needed to achieve 3 or 4 reductions of spores (based on $t_{\Delta D}/\Delta$). Considering limited inactivation of spores at the initial stages of heating (Supplementary Fig. 2), or even slight activation (Supplementary Fig. 1; Finley and Fields, 1962), care must be taken with the interpretation of time needed to achieve only one or two $\log_{10}$ reductions; this time may be a bit longer than just one or two times the D-value, respectively, as D-values do not take into consideration initial heat activation effects on survivor curves.

In conclusion, this study provides information to improve enumeration of *G. stearothermophilus* spores, namely by using TSA medium for plating followed by incubation at 55 °C. In addition, the most important factors that determine variability in spore heat resistance were established, namely the medium used during spore formation and the strains used. For production of heat resistant spores for validation tests, it can be recommended to use strain 9A20 or 4112, and NA CaKMgMn as a sporulation medium with incubation at 55 °C. The data presented in this study furthermore provide insight in the potentially limited inactivation of high level heat resistant *G. stearothermophilus* spores during the production of commercially sterile products using sterilization and UHT heat treatments.

Declaration of interest

None.

Acknowledgements

This research was funded by the TI Food and Nutrition, a public-private partnership on pre-competitive research in food and nutrition. The funding organization had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors would like to thank Han Straatsma for contribution to the data analysis, and Dr. Stella Planchon for providing the CTCPA strains.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.11.005.

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