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Comparative study on the impact of equally stressful environmental sporulation conditions on thermal inactivation kinetics of *B. subtilis* spores

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ABSTRACT

Control of bacterial spores continues to be one of the main challenges for the food industry due to their wide dissemination and extremely high resistance to processing methods. Furthermore, the large variability in heat resistance in spores that contaminate foods makes it difficult to establish general processing conditions. Such heterogeneity not only derives from inherent differences among species and strains, but also from differences in sporulation environments that are generally ignored in spores encountered in foods. We evaluated heat inactivation kinetics and the thermodependency of resistance parameters in *B. subtilis* 168 spores sporulated at adverse temperatures, water activity (a_w), and pH, applying an experimental approach that allowed us to quantitatively compare the impact of each condition. Reduction of incubation temperature from the optimal temperature dramatically reduced thermal resistance, and it was the most influential factor, especially at the highest treatment temperatures. These spores were also more sensitive to chemicals presumably acting in the inner membrane. Reducing sporulation a_w increased heat resistance, although the magnitude of that effect depended on the solute and the treatment temperature. Thus, changes in sporulation environments varied $3D_{100^\circ C}$ values up to 10.4-fold and z values up to 1.7-fold, highlighting the relevance of taking such a source of variability into account when setting heat processing conditions. UV-C treatment and sodium hypochlorite efficiently inactivated all spore populations, including heat-resistant ones produced at low a_w.

1. Introduction

Spore-forming bacteria are important agents of food spoilage, leading to substantial economic losses; furthermore, certain strains of *Bacillus* spp. and *Clostridium* spp. can cause severe foodborne diseases. Control of bacterial spores is still one of the main challenges in food preservation due to their broad dissemination in natural environments, their presence in most raw materials and ingredients, and their capability to survive most food processing methods due to their unique composition and structure (André et al., 2017; Wells-Bennik et al., 2016).

Thermal treatment at elevated temperatures (> 100 °C) is currently the method most widely implemented to inactivate spores for purposes of sterilizing commercial food. Heat is believed to eventually cause spore death by damaging key core protein/s, the identity of which is still cryptic (Coleman et al., 2010, 2007), although other components also play a role in spore resistance (Setlow and Christie, 2021). The low water content of the spore core, together with the presence of high amounts of dipicolinic acid (DPA) chelated with Ca^{+2} and other minerals, likely protects proteins from denaturalization (Beaman and Gerhardt, 1986; Setlow et al., 2006). Furthermore, DNA saturation with small α/β-type acid-soluble spore proteins (SASP) has been shown to provide resistance to heat, despite the fact that wet heat does not primarily cause damage to genetic material (Popham et al., 1995). The thickness and peculiar structure of the peptidoglycan cortex may be involved in keeping the core dehydrated, apart from protecting it from physical stress (den Besten et al., 2018). The coat composition and degree of cross-linking can also contribute to increased heat resistance (Abhyankar et al., 2016; Bressuire-Isoard et al., 2016; Isticato et al., 2020; Melly et al., 2002). It has been recently suggested that properties of the inner membrane may be associated with heat resistance, as variations in spore inactivation among *Bacillus* spp. strains have been related to the presence of a varying number of *spoVA*^{2mob} operons (Berendsen et al., 2016a, 2016b) in which the *2duf* gene, likely encoding a protein located in the inner membrane, is required for increased survival to heat and to other agents targeting this structure in *B. subtilis*

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(Kanaan et al., 2022).

To design effective thermal treatments, it is essential for researchers to acquire a thorough grasp of the impact of heat on spores and to obtain in-depth knowledge of inactivation kinetics. Nevertheless, it remains difficult to make accurate predictions in food processing because contaminating spores tend to display a wide variety of heat resistance behaviors (den Besten et al., 2018). Such variability partially derives from differences in the resistance properties inherent among species and strains (den Besten et al., 2017; Wells-Bennik et al., 2019). This is the main reason why heat processing parameters are often set at a conservative level when attempting to achieve a significant number of logarithm reductions of the most harmful and/or resistant strain likely to contaminate a certain food (Salgado et al., 2011; den Besten et al., 2018; Reddy et al., 2020). Furthermore, in each specific spore strain, environmental conditions during the sporulation process exert a considerable effect on its resistance to heat (Bressuire-Isoard et al., 2018). Sporulation temperature is regarded as the most influential environmental factor playing a role in spore heat resistance, but changes in pH, water activity (a_w), and aeration, as well as composition and physical state of the medium, have also been shown to exert a significant impact (Abbas et al., 2014; Baweja et al., 2008; Cazemier et al., 2001; Nguyen Thi Minh et al., 2011; Rose et al., 2007). From a practical perspective, it is important to evaluate the impact of sporulation conditions on spore resistance in order to appropriately control the inactivation of spores present in foods, particularly in view of the large variety of sporulation niches already prevalent in nature. Soils, as well as the gastrointestinal tracts of animals and insects (which are, in turn, highly diverse and dynamic ecological niches), are regarded as the main sources of sporulated forms, from which spores spread to raw material and ingredients (Carlin, 2011; Gauvry et al., 2021). In addition, food processing facilities are further key sources of spores, since some species are able to adhere to contact surfaces and to food residues, thereby eventually releasing spores (Gauvry et al., 2017; Wijman et al., 2007).

Although researchers tend to consider strain-to-strain variability for heat process calculations using probabilistic approaches (den Besten et al., 2018; Salgado et al., 2011) or using stochastic and kinetic models (Zwietering et al., 2021), such assessments tend to overlook variations that have their origin in environmental sporulation conditions, partly due to a lack of robust data (Carlin, 2011; Ghosh and Setlow, 2009). Although several studies have examined the impact of sporulation conditions on spore heat resistance (Baril et al., 2012; Baweja et al., 2008; Bressuire-Isoard et al., 2018; Garcia et al., 2010; González et al., 1999; Melly et al., 2002; Palop et al., 1999b), there are some disagreements regarding the direction and magnitude of the effect of each factor derived not only from the strain used but also from the experimental setup, which precludes comparison of the influence of different environments gathering literature data. Our study thus aims to assess the effect of several environmental sporulation factors (temperature, a_w, and pH) on heat inactivation kinetics and on the thermodependency of resistance parameters, using *B. subtilis* 168 spores as a model organism. In order to achieve a rational comparison of the impact exerted by different environments, each studied condition was varied up to levels causing the same amount of stress in terms of sporulation efficiency. We also examined the resistance of spore populations to UV-C radiation and sodium hypochlorite, since these two methods are often used for the decontamination of food and contact surfaces with sporicidal intent (Fu et al., 2012; Pineau et al., 2022).

2. Material and methods

2.1. Obtaining and purifying of spore suspensions

B. subtilis 168 was used throughout this study. The strain was maintained at −80 °C in nutrient broth No. 2 (NB; Oxoid, Basingstoke, UK) supplemented with 25 % glycerol. For revitalization, cells were streaked on nutrient agar (Oxoid) supplemented with 0.6 % yeast extract (Oxoid) (NAYE) and incubated at 37 °C for 24 h. For sporulation, a single colony was inoculated into a 60-mL flask containing 10 mL of NB and incubated at 37 °C overnight with shaking (130 rpm). Subsequently, a volume of 20 μL from the culture was inoculated into a 250-mL flask containing 20 mL of liquid 2 × SG sporulation medium. This was composed of 10 g/L bacteriological peptone (Oxoid), 6 g/L Lab Lemco (Oxoid), 2 g/L KCl (Panreac, Barcelona, Spain), and 0.5 g/L $MgSO_4$ (Panreac), supplemented to a final concentration with 1 mM $Ca(NO_3)_2$ (Panreac), 0.1 mM $MnSO_4$ (Carlo Erba, Barcelona, Spain), 1 mM $FeSO_4$ (Sigma-Aldrich, St. Louis, MO, USA) and 0.1 % glucose (Panreac) after autoclaving (Leighton and Doi, 1971). To study the effect of sporulation temperature, flasks were incubated from 18 °C to 45 °C with shaking (130 rpm). To evaluate the impact of a_w, 2 × SG was supplemented with NaCl (Panreac) or glycerol (Panreac) until reaching a_w levels ranging from 0.98 to 0.95. The addition of other solutes such as glucose, sorbitol, or mannitol to a_w 0.98 decreased sporulation to levels below 1000 spores/mL (limit of quantification); these solutes were therefore discarded for further research. The initial pH of 2 × SG was 7.0 and it increased to approximately 9.0 by the end of sporulation. Thus, to study the effect of sporulation pH, the medium was buffered adding a final concentration of 125 mM of MES (pH 5.0; Sigma-Aldrich) or 250 mM of CAPSO (pH 9.0; Sigma-Aldrich). After sporulation, the pH of MES- and CAPSO-buffered cultures changed to final values of 5.7 and 8.8, respectively. Acidity was measured using a pH meter BASIC 20 (Crison Instrument, Barcelona, Spain), while a_w was measured at room temperature with a Decagon CX-1 (Decagon Devices Inc., Pullman, WA, USA). Sporulation at a_w and pH values lying outside of the optimal ones (a_w 0.99, pH 7.0) was performed at 37 °C.

Sporulation was monitored every 24 h by plating aliquots of the cultures previously exposed to a thermal treatment (75 °C, 15 min) and by phase contrast microscopy (Nikon Eclipse E400, Tokyo, Japan). Sporulation was terminated after obtaining two equal consecutive counts under each environmental condition and verifying that longer incubation times did not significantly increase sporulation efficiency. The sporulation efficiency was calculated as the ratio between the plate-count before (vegetative cells plus spores) and after (spores) the heat treatment.

For spore harvest, cultures were centrifuged at 3345 *g* for 20 min at 4 °C and the pellets were washed three times with sterile distilled water. Spores were then purified by buoyant density centrifugation using Nycodenz® as described by previous authors (Ghosh and Setlow, 2009). In brief, cell suspensions were centrifuged and resuspended in a solution of 20 % (w/v) Nycodenz® in PBS (Sigma-Aldrich) at a concentration of ca. 2×10^9 spores/mL. Subsequently, 200 μL were layered steadily on top of 2.5 mL of 50 % (w/v) Nycodenz® in 15-mL conical tubes and centrifuged in a swinging bucket rotor at 3345 *g* for 45 min at 4 °C without brakes. The upper layer where most vegetative cells and debris remained was discarded, and the pellet was carefully recovered and washed several times with distilled water at 6000 *g* for 2 min at room temperature. Spore purity (99 % bright spores) was verified by phase contrast microscopy and the suspensions were kept at −20 °C until use. To assess biological variability, three different spore populations were obtained under each environmental condition.

2.2. Thermal treatment

Heat treatments were carried out in a specially designed thermoresistometer (Condón et al., 1993). In short, this instrument consists in a 450 mL stainless steel chamber equipped with an electrical heater, a refrigeration coil, and an agitation device to ensure inoculum distribution and homogeneity of temperature. The treatment chamber was pressurized with N_2 (2.5 bars) to perform treatments at temperatures over 100 °C and to facilitate sample extraction over time. After stabilization of the target temperature (± 0.1 °C), spores were injected in the treatment chamber containing McIlvaine citrate-phosphate buffer of pH 7.0 (Dawson et al., 1986) to a final concentration of ca. 5×10^4 CFU/mL.

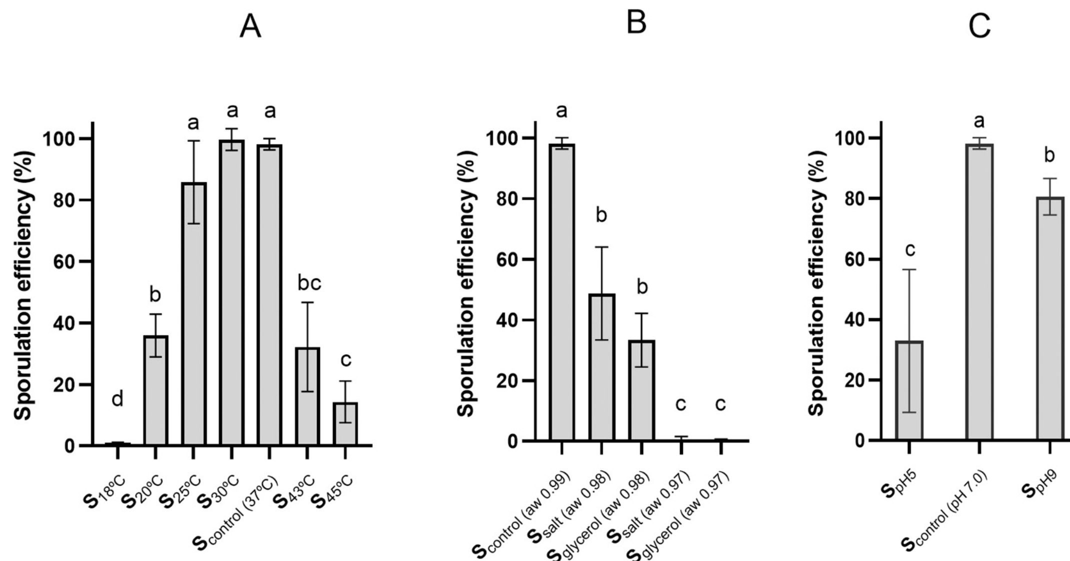

Fig. 1. Influence of incubation temperature (A), a_w (B), and pH (C) in the sporulation efficiency of *B. subtilis* 168. Different letters indicate statistically significant differences ($P \leq 0.05$) in sporulation efficiency among different environmental conditions within each panel.

After inoculation, 0.2-mL samples were extracted at different times and viability was determined as described below.

2.3. Resistance to sodium hypochlorite and dodecylamine

For sodium hypochlorite treatment, spores were diluted in PBS with 2.5 g/L NaClO (Sigma-Aldrich; pH 11.0) to a final concentration of ca. 1.5×10^7 CFU/mL and incubated at 25 °C for 38–40 min with shaking (Cortezzo et al., 2004). For dodecylamine treatment, spores were diluted at the same concentration in 25 mM Tris-HCl buffer of pH 9.0 (Sigma-Aldrich) supplemented with 250 mM NaCl, 5 % DMSO (Sigma-Aldrich) and 1 mM dodecylamine (Sigma-Aldrich), then incubated at 45 °C for 3 h (DeMarco et al., 2021). After treatment, spores were immediately diluted 1/10 in PBS supplemented with sodium thiosulphate (10 g/L; Sigma-Aldrich) or SDS (275 μg/mL; Sigma-Aldrich) to neutralize sodium hypochlorite and dodecylamine, respectively (Cortezzo et al., 2004; DeMarco et al., 2021). Subsequently, serial decimal dilutions were performed and viability was determined as described below.

2.4. UV-C resistance

UV-C treatment was carried out as described in Guillén et al. (2020). In brief, spores were diluted in McIlvaine buffer of pH 7.0 to a concentration of ca. 10^5 CFU/mL, and a volume of 0.2 mL was placed in a microtiter plate sealed with 2 films of vinyl acrylate (Sigma-Aldrich). The microtiter plate was exposed to an irradiance (254 nm) of 0.30 ± 0.02 mW/cm^2 by a 32-W UV-C lamp (VL-208G, Vilber, Germany) located at a distance of 19 cm for 3.5 min under static conditions, resulting in an applied UV-C dose of approximately 63 mJ/cm^2. Irradiance was measured with a UVX radiometer (UVP, LLC, Upland, CA, USA). Temperature of samples did not exceed 30 °C throughout the treatment.

2.5. Determination of survival and sublethal injury

Survival was routinely determined by pour-plating in NAYE. To determine the extent of sublethal injury, spores were also recovered in NAYE supplemented with 5.5 % (*w/v*) of NaCl (NAYE-NaCl), which is the maximum non-inhibitory NaCl concentration for *B. subtilis* 168 determined in previous experiments with non-treated cells (data not shown). NAYE plates were incubated at 37 °C for 24 h, while NAYE-NaCl plates were incubated for 48 h. Longer incubation times did not affect the survival counts. Plate counts were obtained using an automatic colony counting system by image analysis. The survival fraction was calculated as the difference between the logarithm of N_t and N_0 (log (N_t/N_0)), which represent the number of survivors in CFU/mL after different treatment times and prior to treatment, respectively. The number of sublethally injured cells was determined by calculating the difference between the extent of survival in the non-selective (NAYE) and in the selective medium (NAYE-NaCl).

2.6. Calculation of heat resistance parameters

Survival curves to heat were obtained by plotting the survival fraction (log (N_t/N_0)) versus treatment time at each temperature. Since the survival curves had shoulders, experimental data were fitted to the Log-linear + shoulder equation propounded by Geeraerd et al. (2000) (Eq. (1)), using the GInaFiT Excel tool (Geeraerd et al., 2005; KU Leuven, Leuven, Belgium). This equation describes survival curves by means of two parameters: shoulder length (*Sl*, min), defined as the time required to reach the exponential inactivation rate, and the inactivation rate (k_{max}, min^{-1}), defined as the slope of the exponential section of the survival curve. The GInaFiT software also provides the R^2 and root mean square error (RMSE) to assess goodness of fit.

$$Log\ N_t = Log\ N_0 - \frac{k_{max}\ t}{Ln10} + Log\left(\frac{e^{k_{max}\ Sl}}{1 + (e^{k_{max}\ Sl} - 1)e^{-k_{max}\ t}}\right) \quad (1)$$

For comparison, we calculated the time needed for 3 log reductions ($3D_T$) for each treatment temperature. In order to quantify the effect of treatment temperature on heat resistance, thermal death time (TDT) curves were obtained by plotting the logarithm of $3D_T$ values against temperature. *z* values, defined as the number of degrees required to reduce the $3D_T$ value by one logarithmic unit, were calculated as the inverse of the slope of those TDT curves.

2.7. Statistical analysis

Statistical analyses (Student's *t*-test) were performed using GraphPad PRISM 5.0 (GraphPad Software Inc., San Diego, CA, USA), and differences were regarded as significant when *P* was ≤0.05. Data in the figures correspond to averages and standard deviations calculated from at least three biological replicates.

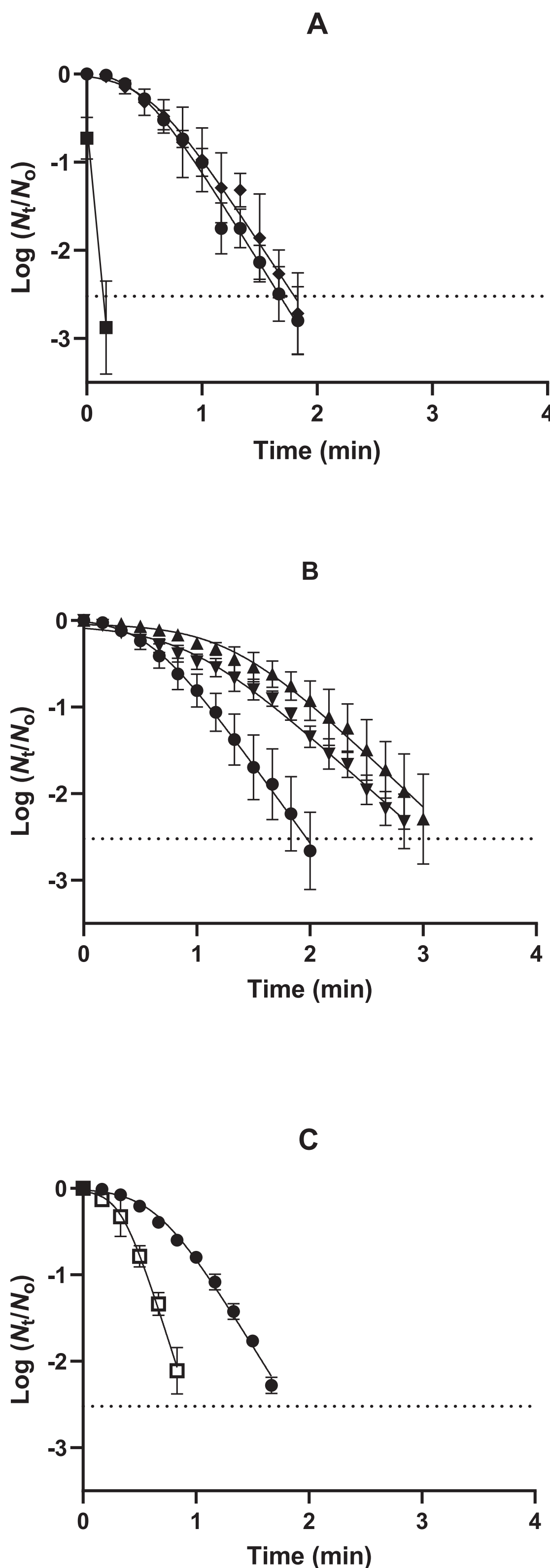

Fig. 2. Survival curves at 105.0 °C of spore populations obtained at different temperatures (A: $S_{20°C}$ (■), $S_{43°C}$ (♦), and $S_{control}$ (●)), a_w (B: S_{gly} (▼), S_{salt} (▲), and $S_{control}$ (●)) and pH (C: S_{pH5} (□) and $S_{control}$ (●)). Survivors were recovered in the non-selective medium (NAYE). Solid lines show predicted data of the Log-linear + shoulder model (Geeraerd et al., 2000) and the dotted line represents the limit of quantification (30 CFU/plate).

3. Results and discussion

3.1. Impact of environmental conditions on sporulation efficiency

Fig. 1 shows the reduction in sporulation efficiency of *B. subtilis* 168 when optimal environmental conditions varied in terms of temperature, a_w or pH ($S_{control}$; 37 °C, a_w 0.99, pH 7.0). Sporulation efficiency was estimated as the survival ratio before and after a pasteurization treatment. Notably, under all tested conditions there were no significant variations in the maximal count of vegetative cells, so that final spore counts could be used as an indicator of the proportion of cells able to sporulate. While sporulation temperatures ranging between 25 °C and 37 °C led to the same ($P > 0.05$) spore concentration, sporulation at 20 °C and 43 °C reduced the efficiency from ca. 99 % to around 34.1 %; further temperature changes to 18 °C and 45 °C reduced sporulation efficiency to 1.0 % and 14.3 %, respectively (Fig. 1). This is partially in accordance with the reduction in sporulation efficiency reported by Gauvry et al. (2021) in *B. subtilis* BSB1, who observed that the final spore counts decreased by 76.1 % in cultures sporulated at 20 °C and by 97.0 % in those incubated at 45 °C compared to the ones sporulated at 37 °C, although these authors did note a significant decrease in spore concentration at 30 °C. Nguyen Thi Minh et al. (2011) observed that spore counts reduced by 97.8 % and by 92.0 % when shifting the sporulation temperature from 37 °C to 19 °C and 45 °C, respectively, in *B. subtilis* ATCC 31324.

Depressing a_w to 0.98 with either glycerol or NaCl led to a similar decrease ($P \leq 0.05$) in sporulation efficiency to ca. 41.1 % (Fig. 1). Sporulation at a_w 0.97 with the two solutes decreased sporulation efficiency by around 99 %, and lower a_w levels ($\leq$ 0.96) resulted in spore concentrations inferior to 1000 spores/mL (data not shown). Widderich et al. (2016) observed that spore counts decreased by ca. 70 % in *B. subtilis* JH642 when sporulated with the same NaCl concentration (0.5 M) that we used to attain a_w 0.98. However, the a_w boundary for sporulation that we observed (a_w 0.96) was higher than that reported by other authors. Nguyen Thi Minh et al. (2011) and Gauvry et al. (2021) demonstrated that *B. subtilis* (strain ATCC 31324 and BSB1, respectively) could sporulate at a_w levels as low as 0.95 with glycerol or NaCl, but with a notably reduced spore yield (by ≥90 %). Such a variability in the a_w sporulation boundary may be explained by differences in the strain's degree of osmotolerance and/or in the composition of the sporulation medium.

Sporulation efficiency decreased ($P \leq 0.05$) when the pH of the sporulation medium was acid (pH 5.0) or alkaline (pH 9.0), up to values of 19.3 % and 80.7 %, respectively (Fig. 1). The pH values we selected were in accordance with growth boundaries previously reported (Gauvry et al., 2021; Mazas et al., 1997); intermediate values (between 5.0–7.0 and 7.0–9.0) could not be examined due to variations in pH at the end of the sporulation process. Mazas et al. (1997) reported a lower reduction in spore efficiency at pH 8.0 (by 5 %–20 %) than at pH 5.5 (by 90 %–95 %) in various *B. cereus* strains, whereas Gauvry et al. (2021) observed similar detriments under both alkaline (8.8) and acidic (5.0) conditions in *B. subtilis* BSB1, with log reduction counts of around 2.5 and 2.0, respectively.

This decrease in sporulation efficiency might be explained by the fact that vegetative cells activate Sigma-B-controlled general stress response and other stress-specific adaptation mechanisms when growing under hostile environmental conditions; in so doing, they somehow reduce sporulation efficiency in response to nutrient starvation, likely as a way to save energy and prevent a scenario in which sporulation cannot be completed after commitment (Reder et al., 2012; Méndez et al., 2004; Widderich et al., 2016). Sporulation efficiency can therefore serve as an indicator of the stress level suffered by cells. Based on this assumption, we opted to compare the impact of different sporulation environments on spore resistance properties, selecting conditions that equally reduced sporulation efficiency to between 32.2 % and 48.7 %. For further research, we thus used spore populations obtained at a low (20 °C,

Table 1

Heat resistance parameters (*Sl*, k_{max}, and $3D_T$) of the different spore populations obtained from the fit of the Log-linear + shoulder model (Geeraerd et al., 2000) to survival curves at different treatment temperatures (Fig. 2, S1, S2, and S3). Survivors were recovered in the non-selective medium (NAYE). Data in brackets represent the standard deviations of the means. Different letters indicate statistically significant differences ($P \leq 0.05$) in resistance parameters among spore populations within each treatment temperature. Values in bold indicate statistically significant differences ($P \leq 0.05$) in resistance parameters between spores obtained at adverse environmental conditions ($S_{20^\circ C}$, $S_{43^\circ C}$, S_{salt}, S_{gly}, and S_{pH5}) and those obtained at the optimal ones ($S_{control}$).

T (°C)	Spore population	*Sl* (min)	k_{max} (min^{-1})	$3D_T$ (min)	R^2	RMSE
105.0	$S_{control}$	0.48^{a} (0.09)	4.66^{a} (0.29)	1.96^{a} (0.10)	0.991	0.113
	$S_{20^\circ C}$	N.D.	N.D.	N.D.	N.D.	N.D.
	$S_{43^\circ C}$	0.57^{a} (0.21)	5.01^{a} (0.63)	1.87^{a} (0.39)	0.990	0.103
	S_{gly}	**0.81^{b} (0.14)**	**2.41^{b} (0.09)**	**3.02^{b} (0.27)**	0.986	0.092
	S_{salt}	**1.28^{c} (0.20)**	**3.01^{c} (0.28)**	**3.60^{b} (0.30)**	0.988	0.089
	S_{pH5}	0.28^{a} (0.14)	**9.12^{d} (1.49)**	**1.09^{c} (0.15)**	0.985	0.168
100.0	$S_{control}$	3.13^{a} (0.32)	1.32^{a} (0.01)	8.24^{a} (0.34)	0.988	0.145
	$S_{20^\circ C}$	**0.43^{b} (0.10)**	**6.59^{b} (0.71)**	**1.49^{b} (0.40)**	0.987	0.15
	$S_{43^\circ C}$	3.36^{a} (0.20)	1.19^{ac} (0.09)	9.18^{ac} (0.67)	0.985	0.114
	S_{gly}	**4.35^{c} (0.25)**	**1.10^{c} (0.01)**	**10.19^{c} (0.85)**	0.988	0.123
	S_{salt}	**6.30^{d} (0.26)**	**0.75^{d} (0.01)**	**15.49^{d} (0.12)**	0.976	0.089
	S_{pH5}	**1.56^{e} (0.51)**	**2.30^{e} (0.75)**	**4.71^{e} (0.42)**	0.967	0.207
97.5	$S_{control}$	7.33^{a} (1.05)	0.56^{a} (0.02)	18.90^{a} (1.66)	0.993	0.120
	$S_{20^\circ C}$	**0.91^{b} (0.24)**	**1.55^{b} (0.51)**	**5.06^{b} (1.21)**	0.957	0.229
	$S_{43^\circ C}$	7.99^{a} (0.67)	0.59^{ac} (0.22)	17.31^{a} (2.53)	0.972	0.239
	S_{gly}	7.93^{a} (0.99)	0.62^{a} (0.06)	19.25^{a} (1.58)	0.988	0.152
	S_{salt}	**12.49^{c} (1.45)**	**0.43^{c} (0.08)**	**28.57^{c} (0.79)**	0.961	0.151
	S_{pH5}	**3.16^{d} (1.26)**	**1.30^{b} (0.45)**	**9.52^{d} (0.88)**	0.990	0.137
92.5	$S_{20^\circ C}$	5.42 (1.43)	0.25 (0.04)	33.4 (4.92)	0.982	0.061

denominated as $S_{20^\circ C}$) and high (43 °C, denominated as $S_{43^\circ C}$) temperature, low pH (pH 5.0, denominated as S_{pH5}) and low a_w (0.98 with either glycerol or NaCl, denominated as S_{gly} and S_{salt}, respectively), and, for comparison, populations sporulated under control conditions (denominated as $S_{control}$).

3.2. Impact of sporulation conditions on spore heat resistance

To characterize thermal resistance of spores obtained at adverse temperatures, a_w, or pH in comparison with those obtained at optimal sporulation conditions ($S_{control}$), survival curves at three different temperatures (97.5 °C, 100.0 °C, and 105.0 °C) were obtained (Fig. 2, Fig. S1 and S2). Spores obtained at 20 °C ($S_{20^\circ C}$) were also treated at 92.5 °C due to their elevated sensitivity at 105.0 °C (Fig. S3). As observed, most inactivation curves presented a shoulder phase prior to exponential decay. In order to estimate the influence of environmental sporulation conditions on shoulder length and inactivation rate, survival curves were fitted to the Log-linear + shoulder model developed by Geeraerd et al. (2000). Table 1 displays the thermal resistance parameters (*Sl* and k_{max} values) and the time for achieving 3 log reductions ($3D_T$ value) obtained for each sporulation condition and treatment temperature. To evaluate the impact of treatment temperature on the heat resistance of each spore population, we constructed thermal death time (TDT) curves from which we calculated *z* values expressing the effect of temperature changes on $3D_T$ values (Fig. 3, Table 2).

Spores obtained at the lowest temperature ($S_{20^\circ C}$) were the least heat-resistant populations at all temperatures tested. At 105 °C, survival counts of $S_{20^\circ C}$ fell below the limit of quantification (2.5 log units) after the first 10 s of treatment, whereas an inactivation of the control populations to the same extent required around 1.7 min (Fig. 2A). At 100.0 °C and 97.5 °C, $3D_T$ values of $S_{20^\circ C}$ were 5.5- and 3.8-fold lower ($P \leq 0.05$) than those of the controls (Table 1, Fig. S1 and S2). Such hypersensitivity of $S_{20^\circ C}$ spores was due to the shortening of the *Sl* and to a notably high k_{max} in comparison to the controls (Table 1). Consequently, TDT curves of $S_{20^\circ C}$ had a more pronounced slope, with *z* values 2.0 °C lower ($P \leq 0.05$) than $S_{control}$ (Fig. 3, Table 2). In contrast, even though an increase in incubation temperature from 37 °C to 43 °C reduced sporulation efficiency to a similar extent as decreasing temperature to 20 °C (Fig. 1), $S_{43^\circ C}$ spores showed $3D_T$ and *z* values that were similar ($P > 0.05$) to those sporulated at 37 °C (Tables 1 and 2).

It has been widely demonstrated that sporulation temperature has a considerable impact on spore heat resistance: D_T values can vary up to 10-fold depending on the incubation temperature (Bressuire-Isoard et al., 2018; Palop et al., 1999a). However, the range of temperatures that exert the most influence can vary among species and strains (Palop et al., 1999a). As noted in our results, heat resistance of *Bacillus* spp. spores generally decreases when sporulation temperature is reduced from the optimum (Baril et al., 2012; Baweja et al., 2008; Bressuire-Isoard et al., 2018; Garcia et al., 2010; González et al., 1999; Lindsay et al., 1990; Melly et al., 2002; Planchon et al., 2011). The higher sensitivity of spores produced at suboptimal temperatures has been attributed to increased core water content, as well as to decreased mineral and DPA content (Baweja et al., 2008; Beaman and Gerhardt, 1986; Melly et al., 2002; Palop et al., 1999b). Furthermore, variations in heat resistance induced by sporulation temperature have been linked to changes in the structure and composition of the cortex peptidoglycan and the coat (Bressuire-Isoard et al., 2016; Isticato et al., 2020; Melly et al., 2002). Conversely, as corroborated by our findings in spores obtained at high temperature ($S_{43^\circ C}$), rising sporulation temperature does not systematically result in spores with increased resistance – or it only does so up to a certain value, to which certain strains even display a substantial degree of sensitisation (Baril et al., 2012; Garcia et al., 2010; González et al., 1999; Lindsay et al., 1990; Mtimet et al., 2015; Palop et al., 1999b).

Several authors have reported that *z* value is a specific feature of each strain independently of sporulation temperature (Baril et al., 2012; González et al., 1999; Raso et al., 1995; Sala et al., 1995). As opposed to those findings, our observations indicate that *z* value changes when *B. subtilis* sporulate at 20 °C, as compared to optimal and supra-optimal sporulation temperatures. This discrepancy might be associated with the range of sporulation temperatures tested. To the best of our knowledge, the current study is the first one to report the influence of sporulation temperature, using equally stressful high and low incubation temperatures (i.e., reducing sporulation efficiency to ca. 34 %, Fig. 1), on heat inactivation kinetics of spores and its thermodependency. Furthermore, this might depend on strain-to-strain variations and on the composition of the sporulation medium. As such, it has been reported that *z* values did vary with sporulation temperature under acidic treatment

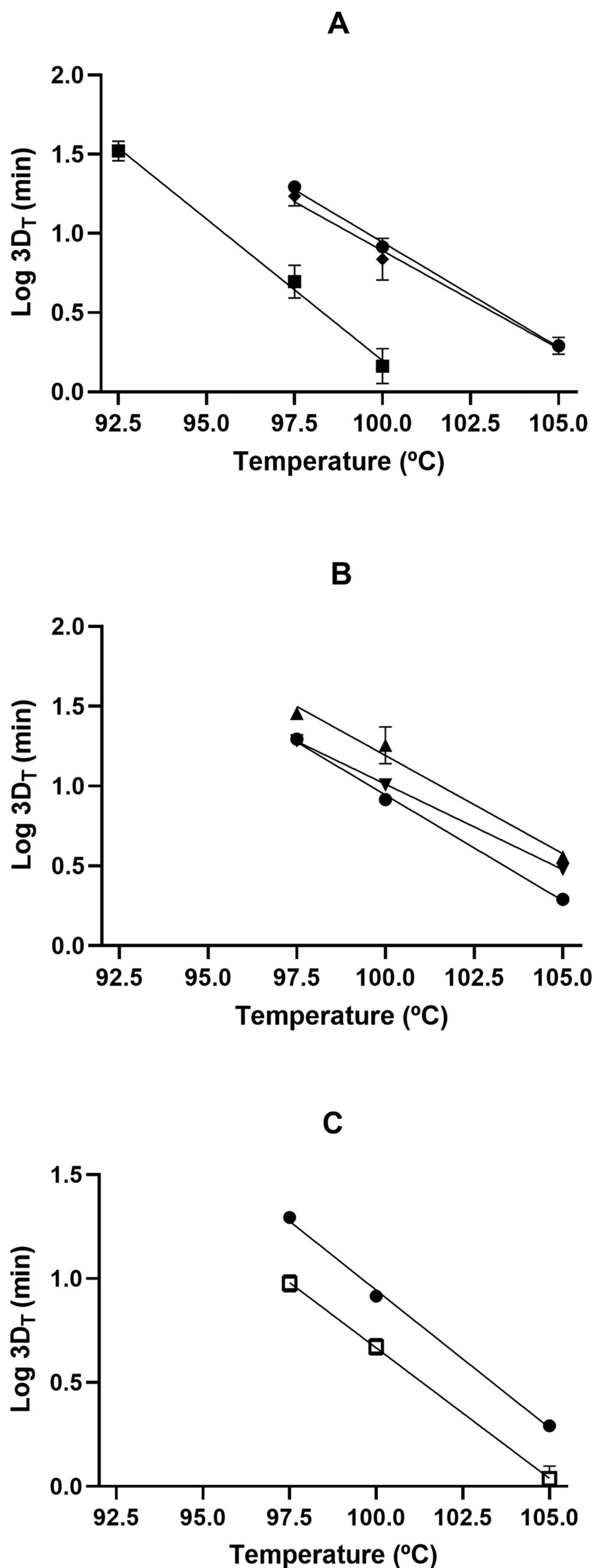

Fig. 3. Thermal death time (TDT) curves, plotting 3D$_T$ values (min) against treatment temperature, of the spore populations obtained at different temperatures (A: $S_{20°C}$ (■), $S_{43°C}$ (♦), and $S_{control}$ (●)), a_w (B: S_{gly} (▼), S_{salt} (▲), and $S_{control}$ (●)) and pH (C: of S_{pH5} (□) and $S_{control}$ (●)).

Table 2

z values calculated from TDT curves (Fig. 3) of the indicated spore populations. Data in brackets represent the standard deviations of the means. Different letters indicate statistically significant differences ($P \leq 0.05$) in *z* values among the different spore populations.

Spore population	*z* (°C)	R^2
$S_{control}$	7.59^{a} (0.08)	0.995
$S_{20°C}$	5.59^{b} (0.12)	0.977
$S_{43°C}$	8.44^{acd} (0.80)	0.958
S_{salt}	8.13^{c} (0.18)	0.997
S_{gly}	9.26^{d} (0.41)	0.991
S_{pH5}	7.32^{a} (0.58)	0.990

conditions (Ruiz et al., 2022; Sala et al., 1995).

Interestingly, spore populations obtained at a_w 0.98 using either glycerol (S_{gly}) or salt (S_{salt}) were more resistant to heat than the controls; nevertheless, the magnitude of that effect depended on the solute and treatment temperature used. At 105.0 °C, S_{gly} and S_{salt} showed 0.3 and 0.8 min longer *Sl*, respectively, and on average 1.7-fold higher k_{max} than $S_{control}$ (Fig. 2, Table 1). Furthermore, the $3D_{105.0°C}$ values estimated for S_{gly} and S_{salt} spores were 1.5- and 1.8-fold higher ($P \leq 0.05$) than those of the controls, respectively (Table 1). While the increased heat resistance of S_{salt} was maintained when decreasing treatment temperature, the differences in 3D$_T$ of S_{gly} compared to the controls progressively decreased. For instance, differences in 3D$_T$ values between S_{gly} and controls were reduced ($P \leq 0.05$) to 1.2-fold at 100.0 °C and practically disappeared ($P > 0.05$) at 97.5 °C (Table 1, Fig. S1 and S2). Thus, the *z* value of *B. subtilis* increased from 7.6 °C to 9.3 °C when sporulating at lowered a_w with glycerol, whereas it increased to 8.1 °C when NaCl was the a_w depressor (Fig. 3, Table 2).

Studies of the influence of sporulation a_w on the thermal resistance of spores are scarce and show contradictory results. In agreement with our findings, Aouadhi et al. (2016) reported that *B. sporothermodurans* LTIS27 sporulated at a_w 0.95 with NaCl presented 3.2-log cycle higher resistance to heat (95 °C, 12 min) in combination with nisin (125 UI/mL) than spores obtained without depressing a_w; however, heat inactivation was not affected when sporulated at a_w 0.95 with glycerol. On the other hand, Jakobsen and Murrell (1977) observed that decreasing the a_w of sporulation medium with NaCl (a_w 0.95) or glycerol (a_w 0.91) did not modify $D_{95°C}$ values of *B. cereus* T spores. Furthermore, Nguyen Thi Minh et al. (2011) showed that *B. subtilis* ATCC 31324 spores obtained at a_w 0.95 with either NaCl or glycerol displayed 2-fold lower $D_{97°C}$ values than those produced in basal medium. Such discrepancies might be attributed to multiple causes, such as differences in the strain's inherent resistance, composition of the sporulation medium, and level of a_w reduction, as well as the solute and treatment temperature used. Regarding the latter, our results show that reducing a_w of sporulation medium can affect *z* values, depending on the solute used, so that the magnitude of changes in heat resistance is strongly dependent on treatment temperature. Further research is therefore necessary in order to truly assess the impact of reducing a_w during sporulation on spore resistance in various strains, using a wide range of different solutes and treatment temperatures.

S_{pH5} spores were more sensitive to heat than $S_{control}$ spores at all temperatures tested, showing *Sl* and k_{max} values lower and higher, respectively, than the latter (Fig. 2, Fig. S1 and S2, Table 1). Consequently, 3D$_T$ values of $S_{pH5.0}$ spores were ca. 2-fold lower ($P \leq 0.05$) than those of the control spores at all temperatures (Table 1), and the two had similar ($P > 0.05$) *z* values (Fig. 3, Table 2). Other studies have likewise found that decreasing sporulation pH from the optimal value tends to decrease thermal resistance while maintaining *z* values (Baril et al., 2012; Mazas et al., 1997; Pang et al., 1983). For instance, Baril et al. (2012) reported that reducing pH of sporulation medium decreased $D_{90°C}$ values of *B. weihenstephanesis* KBAB4 (from pH 7.2 to 5.9) and $D_{100°C}$ values of *B. licheniformis* A0978 (from pH 7.2 to 6.3) by almost half. The higher thermal sensitivity of spores produced at acidic pH has

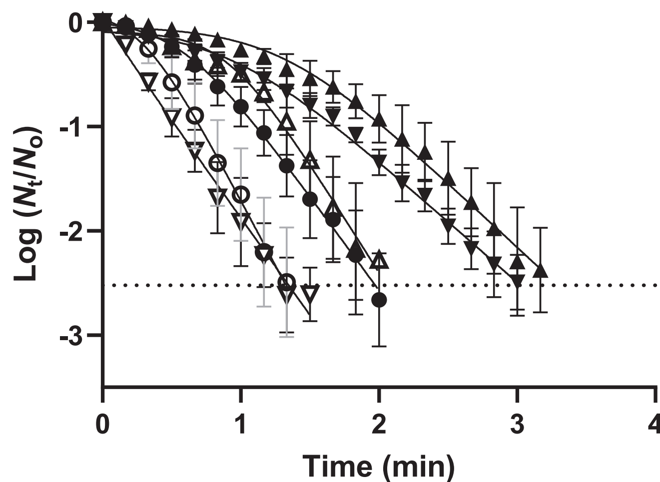

Fig. 4. Survival curves at 105.0 °C of spore populations obtained at different a_w (S_{gly} (▼), S_{salt} (▲), and $S_{control}$ (●)). Survivors were recovered in the non-selective (NAYE, close symbols) and selective medium (NAYE-NaCl, open symbols). Solid lines show predicted data of the Log-linear + shoulder model (Geeraerd et al., 2000) and the dotted line represents the limit of quantification (30 CFU/plate).

been partly attributed to changes in spore mineralization content or the availability of certain transition metals at those pH levels (Alderton and Snell, 1963; Kihm et al., 1990). Moreover, carboxylic groups of the cortex are also protonated, thus ultimately leading to increased water content in the protoplast (Gould and Dring, 1972).

Only few studies have compared the impact of several different environmental factors on spore heat resistance in the same experimental setup (Aouadhi et al., 2016; Baweja et al., 2008; Nguyen Thi Minh et al., 2011): although they applied different stress levels (in terms of sporulation efficiency) for each condition, and they used a single treatment temperature, thus preventing comparison of the thermodependency of heat resistance. Our data illustrate that a reduction of sporulation temperature has a more pronounced effect on thermal resistance than decreasing pH or increasing a_w, to the point of equally reducing sporulation efficiency by 58.9–67.0 %, at all treatment temperatures. Furthermore, this study highlights the paramount importance of environmental sporulation conditions on the variability of thermal resistance of spores from a specific strain. Changes in temperature, pH, and a_w provoked variations up to 10.4-fold in $3D_{100^\circ C}$ values (comparing values from $S_{20^\circ C}$ and S_{salt}) and up to 1.7-fold in z values (comparing values from $S_{20^\circ C}$ and S_{gly}) in spores of *B. subtilis* 168 (Tables 1 and 2). Further studies are needed to elucidate whether such a magnitude of variations can be extrapolated to other sporulated strains. The variability we observed in $3D_{100^\circ C}$ values was maintained when calculated $4D_{100^\circ C}$ values (11.1-fold change), and it is superior to that observed in the resistance of different *B. subtilis* strains (without harbouring SpoVA^{2mob} mobile genetic resistance elements) reported by den Besten et al. (2017), where $4D_{100^\circ C}$ values ranged between 4.9 and 25.1 min (5.1-fold change). Therefore, our results underscore that variance in heat resistance induced by environmental sporulation conditions should be as prominently taken into consideration as intraspecific variability in risk assessment for the determination of heat processing conditions designed to ensure appropriate levels of food safety.

3.3. Impact of sporulation conditions on heat-induced sublethal injury

Since a reduction of sporulation a_w increased heat resistance, we investigated the extent of heat-induced sublethal injury in spores obtained at a_w 0.98 and in the corresponding control populations (a_w 0.99) at a treatment temperature of 105 °C (in the temperature at which the largest differences in kinetics were observed) by recovering survivors in a non-selective (NAYE) and a selective (NAYE-NaCl) medium (Fig. 4). Such technique has been used to discriminate sublethally injured spores due to the inability of those with permeabilized membranes to grow (Cazemier et al., 2001; Cortezzo et al., 2004; Coleman and Setlow, 2009). For comparison, we fitted the two survival curves obtained in the non-selective and selective medium to the Log-linear + shoulder model (Geeraerd et al., 2000) as previously described (Table 3).

The shoulder of survival curves of $S_{control}$ spores almost disappeared, and k_{max} decreased 0.8-fold ($P \leq 0.05$) when recovered in the selective medium (Fig. 4, Table 3). This agrees with previous interpretations relating shoulders of heat inactivation kinetics to damage and repair phenomena, suggesting that cell death occurs when spores are unable to repair accumulated damage upon germination (Condón et al., 1996; Feeherry et al., 1987). Both *Sl* and k_{max} obtained from S_{gly} survival curves also changed significantly ($P \leq 0.05$) when plated in NAYE-NaCl, but to a higher extent (Table 3), in such a way that counts in the selective medium practically overlapped with those of the controls (Fig. 4). This suggests that the higher survival presented by S_{gly} spores in the non-selective medium compared to the controls may be related to an improved repair capability of sublethal damage in the former.

On the other hand, survival counts of S_{salt} in NAYE-NaCl were higher than those of $S_{control}$ at all time points (Fig. 4). In fact, inactivation curves of S_{salt} spores in the selective medium presented a shoulder of around 0.8 min, indicating that they accumulated less sublethal injury (Table 3). Furthermore, the proportion of sublethally injured spores for the same level of inactivation in the selective medium was lower in $S_{control}$ than in S_{salt}: the log number of sublethally damaged spores when reaching 2.5 log reductions in NAYE-NaCl (1.3 min of treatment for $S_{control}$ and 2.2 min of treatment in S_{salt}) was 1.2 and 1.6, respectively (Fig. 4).

Altogether, these results evidence that the higher heat resistance of spores obtained at reduced a_w compared to those sporulated at a_w 0.99 might be explained by an improved capability to repair thermal injuries and/or a higher intrinsic resistance to damage, probably occurring in spore membranes detectable in the selective medium (in the case of S_{salt}). In practice, these observations compromise the effectiveness of

Table 3
Heat resistance parameters (*Sl*, k_{max}, and $3D_T$) of S_{salt}, S_{gly}, and $S_{control}$ populations obtained from the fit of the Log-linear + shoulder model (Geeraerd et al., 2000) to survival curves at 105.0 °C obtained in the non-selective (NAYE) and selective medium (NAYE-NaCl) (Fig. 4). Data in brackets represent the standard deviations of the means. Different letters indicate statistically significant differences ($P \leq 0.05$) in resistance parameters among spore populations plated in both NAYE and NAYE-NaCl. Values in bold indicate statistically significant differences ($P \leq 0.05$) in resistance parameters among spore populations obtained at reduced a_w (S_{salt} and S_{gly}) and the ones obtained at the optimal one ($S_{control}$) plated either in NAYE or NAYE-NaCl.

Plating medium	Spore population	*Sl* (min)	k_{max} (min^{-1})	$3D_T$ (min)	R^2	RMSE
NAYE	$S_{control}$	0.48^a (0.09)	4.62^a (0.18)	1.98^a (0.15)	0.991	0.113
	S_{gly}	**0.81^b (0.14)**	**2.61^b (0.14)**	**3.46^b (0.28)**	0.986	0.092
	S_{salt}	**1.28^c (0.20)**	**2.96^b (0.39)**	**3.61^b (0.51)**	0.988	0.089
NAYE-NaCl	$S_{control}$	0.26^a (0.15)	5.35^c (0.42)	1.52^a (0.36)	0.977	0.139
	S_{gly}	N.D.	4.38^{ac} (0.63)	1.60^a (0.25)	0.983	0.135
	S_{salt}	**0.77^b (0.23)**	5.84^{ac} (1.15)	1.98^a (0.45)	0.988	0.095

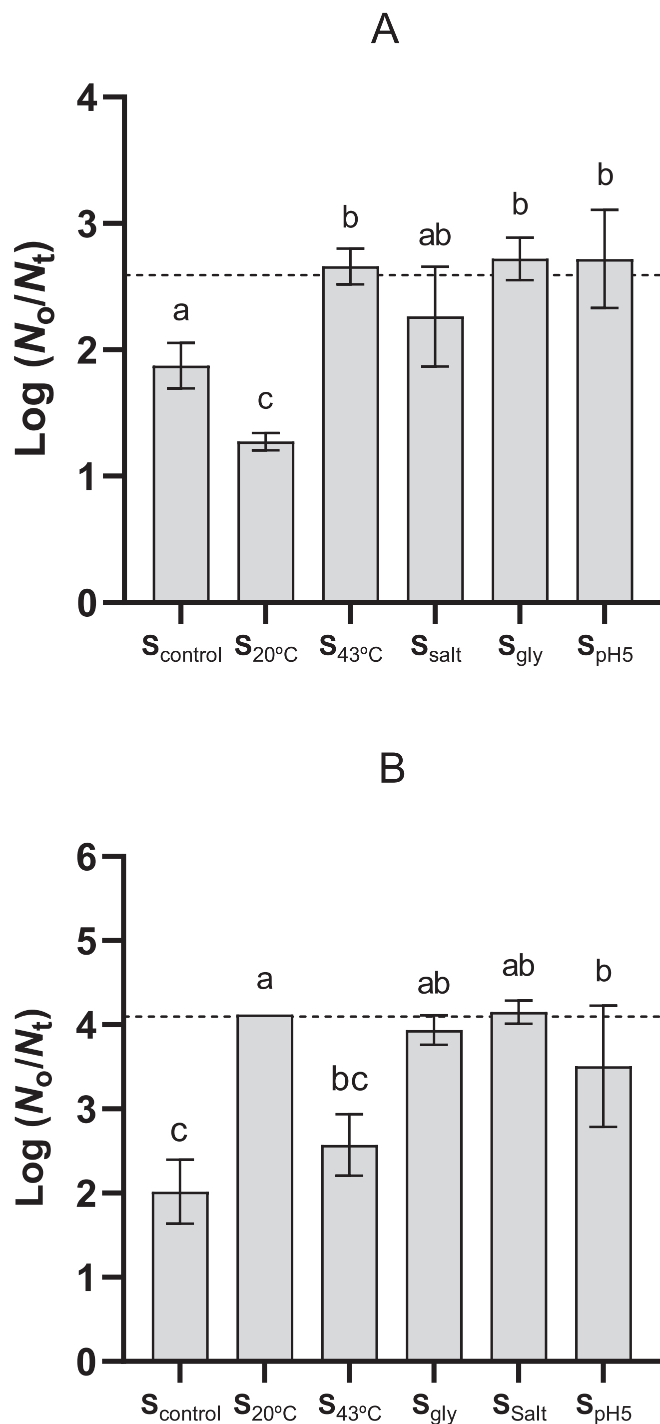

Fig. 5. Survival (expressed as log(N_0/N_t)) of spore populations obtained at different adverse environmental conditions ($S_{20^\circ C}$, $S_{43^\circ C}$, S_{salt}, S_{gly}, and S_{pH5}) and the optimal ones ($S_{control}$) to UV-C treatment (63 mJ/cm^2, 254 nm; A) and to sodium hypochlorite (2.5 g/L, pH 11, 25 °C, 38–40 min; B). Survivors were recovered in the non-selective medium (NAYE). Dotted line represents the limit of quantification (30 CFU/plate). Different letters indicate statistically significant differences ($P \leq 0.05$) in survival among spore populations within each panel.

hurdle preservation approaches that rely on the inactivation of sublethally damaged cells when dealing with spores formed in high salinity environments.

3.4. Impact of sporulation conditions on spore resistance to UV-C radiation and chemicals

As UV-C and sodium hypochlorite are used as alternative food preservation methods against heat and for disinfection of contact surfaces (Fu et al., 2012; Pineau et al., 2022), we examined the resistance of spore populations obtained under adverse environmental conditions to both agents (Fig. 5). Sporulation temperature exerted a pronounced influence on resistance to UV-C radiation (63 mJ/cm^2, 254 nm). While reducing sporulation temperature to 20 °C dramatically reduced heat resistance (Fig. 2), $S_{20^\circ C}$ spores showed higher ($P \leq 0.05$) UV-C resistance than $S_{control}$ spores (Fig. 5A). In contrast, $S_{43^\circ C}$ spores were more UV-C sensitive, so that the difference in inactivation between spores incubated at the supraoptimal and suboptimal temperatures was around 1.5 log cycles. Contrarily, Melly et al. (2002) did not observe UV-C resistance changes in *B. subtilis* PS832 spores produced at 23 °C, 37 °C and 48 °C, and Planchon et al. (2011) observed an only slightly higher resistance displayed by *B. cereus* KBAB4 spores obtained at 30 °C than at 10 °C at a dose of 27 mJ/cm^2. Reducing sporulation a_w tended to decrease UV-C resistance, especially when using glycerol as a solute, since S_{gly} spores were 0.9-log cycle more vulnerable ($P \leq 0.05$) than $S_{control}$ spores (Fig. 5A). Conversely, acidification of the sporulation medium did not affect ($P > 0.05$) UV-C resistance. UV radiation at 254 nm causes damage to the DNA of spores, and the presence of DPA, SASP, and DNA repair enzymes contributes to their resistance (Setlow, 2001, 2006; Setlow and Christie, 2021). Further studies will be needed to mechanistically infer the causes of variations in UV resistance among spore populations.

Regarding resistance to sodium hypochlorite (2.5 g/L, pH 11.0, 38–40 min; Fig. 5B), $S_{20^\circ C}$ spores were the most sensitive populations (inactivation was >3.0 log cycles higher than $S_{control}$ spores), followed by those sporulated at low a_w with either glycerol or salt (2.2 and 2.6 log cycles higher ($P \leq 0.05$) than $S_{control}$ spores, respectively). In addition, S_{pH5} spores were 1.5 log cycles more inactivated ($P \leq 0.05$) than $S_{control}$ populations. In contrast, increasing sporulation temperature to 43 °C did not affect ($P > 0.05$) resistance to hypochlorite, even at shorter or longer treatment times (data not shown). This disagrees with previous observations of Young and Setlow (2003), who demonstrated that *B. subtilis* PS533 spores produced at 46 °C were more resistant than those obtained at 37 °C, which, in turn, were equally resistant as those incubated at 20 °C. Sodium hypochlorite is believed to cause damages of still unknown nature in the inner spore membrane (Cortezzo et al., 2004; Young and Setlow, 2003). However, resistance of spores to dodecylamine (Fig. S4), another compound that probably disrupts inner membrane permeability (Mokashi et al., 2020), did not correlate with hypochlorite inactivation. Whereas $S_{20^\circ C}$ populations were also sensitive to dodecylamine, spores produced at reduced a_w displayed a degree of inactivation similar ($P > 0.05$) to that of $S_{control}$ spores. Therefore, further studies are needed to elucidate the effect of sporulation temperature and a_w on inner membrane properties and their role in the resistance to these chemicals and heat. Changes in coat structure and composition derived from different sporulation temperatures (Melly et al., 2002; Bressuire-Isoard et al., 2016; Isticato et al., 2020) may also be involved in variations in terms of resistance, as the coat acts as a major permeability barrier against hypochlorite and dodecylamine (DeMarco et al., 2021; Mokashi et al., 2020; Young and Setlow, 2003). However, further studies should be performed to ascertain whether differences in the properties of these structures exist.

4. Conclusions

Although the effect of certain environmental factors, such as sporulation temperature, on spore heat resistance has been widely investigated, it remains practically impossible to compare data from different studies due to differences in a series of methodological aspects such as strain, range of sporulation conditions, composition of the sporulation

medium, incubation periods, protocol for spore purification, and heat treatment intensity. The current study is the first one to compare the effect of sporulation temperature, a_w, and pH at equally stressful levels, in terms of reduction in sporulation efficiency, on heat inactivation kinetics of *B. subtilis* 168 spores and their thermodependency using the same experimental setup, thereby allowing us to draw reliable conclusions. Decreasing sporulation temperature from the optimum (from 37 °C to 20 °C) was identified as the most influential factor, especially at the highest treatment temperatures. Contrarily to previous reports, a decrease in sporulation a_w led to an increase in heat resistance, but the range of treatment temperatures where the increment was observed, along with its magnitude, depended on the solute used (glycerol or NaCl). Optimization of thermal sterilization processes should lend greater prominence to this fact, since soil with high salt levels is often one of the most frequent sporulation niches of *Bacillus* spp. and *Clostridium* spp., and soil salinity is expected to increase with climate change (Khamidov et al., 2022). The variability in thermal resistance ($3D_{100^\circ C}$ values) induced by environmental sporulation conditions in *B. subtilis* 168 (10.4-fold; comparing data from spores produced at 20 °C and at a_w 0.98 using NaCl) was greater than that previously reported among *B. subtilis* strains (den Besten et al., 2017); therefore, this source of variability should also be taken into account when determining heat processing conditions. However, more data are necessary on different strains, in order to evaluate whether this observed variability can be extrapolated to other spore-forming bacteria.

Both UV-C radiation and sodium hypochlorite were effectively capable of inactivating the most heat-resistant spores produced at reduced a_w. UV-C technology has been proposed as an effective method for the inactivation of heat-resistant spores, such as *B. subtilis* strains harbouring $SpoVA^{2mob}$ elements (Kanaan et al., 2022), or spores from thermophilic species (Gayán et al., 2013); our results indeed confirm that UV-C can efficiently inactivate spores produced under a series of varying environmental conditions. Although spores produced at low temperatures may be a concern when using UV processing because of their increased resistance, the variation in UV inactivation among spore populations obtained in different environments was much lower than that observed in thermal treatments.

Our study also sheds some light on different spore properties among populations obtained under adverse environmental conditions. Spores produced at the lowest temperature exhibited higher sensitivity to sodium hypochlorite and dodecylamine, pointing out that future work should be focused on the role of the inner membrane changes induced by sporulation temperature in the resistance to these chemicals and heat. However, spores obtained at reduced a_w, despite their increased heat resistance, were sensitive to hypochlorite but not to dodecylamine, thereby indicating that the mechanisms of resistance changes in these populations require further research. It should be noted that the use of either glycerol or NaCl to depress a_w of sporulation medium may provide heat protection via a series of different mechanisms. According to the survival rate obtained by plating in selective and non-selective media, spores produced in the presence of glycerol showed a higher capability of damage repair, while those incubated at high salinity also suffered sublethal injury to a lesser degree. In practice, this should also be taken into account in the development of food preservation strategies relying on hurdle approaches that benefit from sublethal damage of cells to improve microbial inactivation.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2023.110349.

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