Food Research International 182 (2024) 114064

Contents lists available at ScienceDirect

Food Research International

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

Impact of sporulation temperature on germination of *Bacillus subtilis* spores under optimal and adverse environmental conditions

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ARTICLE INFO

Keywords:
Bacterial spores
Bacillus subtilis
Germination kinetics
Germination boundaries
Refrigeration
Water activity
pH
Sporulation temperature

ABSTRACT

Bacillus subtilis spores are important food spoilage agents and are occasionally involved in food poisoning. In foods that are not processed with intense heat, such bacterial spores are controlled by a combination of different hurdles, such as refrigeration, acidification, and low water activity (a_w), which inhibit or delay germination and/or growth. Sporulation temperature has long been regarded as a relevant factor for the assessment of germination in chemically defined media, but little is known about its impact on food preservation environments. In this study, we compared germination dynamics of *B. subtilis* spores produced at optimal temperature (37 °C) with others incubated at suboptimal (20 °C) and supraoptimal (43 °C) temperatures in a variety of nutrients (rich-growth medium, L-alanine, L-valine, and AGFK) under optimal conditions as well as under food-related stresses (low a_w, pH, and temperature). Spores produced at 20 °C had a lower germination rate and efficiency than those incubated at 37 °C in all the nutrients, while those sporulated at 43 °C displayed a higher germination rate and/or efficiency in response to rich-growth medium and mostly to L-alanine and AGFK under optimal environmental conditions. However, differences in germination induced by changes in sporulation temperature decreased when spores were activated by heat, mainly due to the greater benefit of heat for spores produced at 20 °C and 37 °C than at 43 °C, especially in AGFK. Non-heat-activated spores produced at 43 °C still displayed superior germination fitness under certain stresses that had considerably impaired the germination of the other two populations, such as reduced temperature and a_w. Moreover, they presented lower temperature and pH boundaries for the inhibition of germination in rich-growth medium, while requiring a higher NaCl concentration threshold compared to spores obtained at optimal and suboptimal temperature. Sporulation temperature is therefore a relevant source of variability in spore germination that should be taken into account for the accurate prediction of spore behaviour under variable food preservation conditions with the aim of improving food safety and stability.

1. Introduction

Bacterial spores are highly resistant to most mild food processing methods: they can germinate during storage, resume metabolic activity, and multiply, thereby leading to spoilage or foodborne illnesses (André, Vallaeys, & Planchon, 2017; Wells-Bennik et al., 2016). Thus, control of spores in products that cannot be subjected to a sufficient degree of intense thermal treatment to inactivate them without unacceptably lowering the product's quality must rely on a combination of hurdles, such as refrigeration, acidification, low water activity (a_w), and/or the addition of antimicrobial compounds, all of which inhibit or slow down spore germination and/or outgrowth (Bevilacqua, Sinigaglia, & Corbo, 2008; Ciarciaglini et al., 2000; Levinson & Hyatt, 1970; Rao et al., 2018; Smoot & Pierson, 1982). Therefore, to establish adequate hurdle conditions and achieve satisfactory shelf life, knowledge of germination dynamics in foods subjected to preservation stresses is essential. In addition, an in-depth understanding of germination kinetics is required in order to design effective germination-inactivation approaches, which are proposed as alternative spore eradication methods with the aim of achieving food quality that is superior to that of food subjected to intense thermal treatments (Kwon et al., 2022; Løvdal et al., 2011).

An accurate prediction of spore behaviour requires the quantification of the impact of sporulation conditions on germination kinetics. This is extremely important for food preservation because

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https://doi.org/10.1016/j.foodres.2024.114064
Received 20 October 2023; Received in revised form 13 January 2024; Accepted 25 January 2024
Available online 29 January 2024

contaminating spores can originate from a variety of sporulation niches, mainly soil, the gastrointestinal tract of animals, and food processing facilities (Carlin, 2011; Gauvry et al., 2017), all of which, in turn, present fluctuating environmental conditions. Sporulation temperature exerts a considerable influence on germination behaviour, but data reported on the magnitude and direction of its effect are inconsistent (Bressuire-Isoard et al., 2018). Higher as well as lower germination rates and/or efficiency have been observed when either raising or lowering sporulation temperature from the optimum level for *Bacillus* spp. spores, and contradictory results have even been reported using the same strain in independent studies (Gounina-Allouane et al., 2008; Garcia et al., 2010; Planchon et al., 2011; Luu et al., 2015; Bressuire-Isoard et al., 2016; Isticato et al., 2020). This could be attributed to differences in purification methods, maturation times, the range of sporulation temperatures, and varying composition of the sporulation medium (Hornstra et al., 2006; Isticato et al., 2020; Segev et al., 2012). Moreover, the influence of sporulation temperature on germination has mainly been studied under laboratory (optimal) conditions, and therefore results from studies of that nature cannot be readily extrapolated to foods, as foods are normally subjected to several types of preservation stresses.

In view of this lack of knowledge, our study aimed to compare the impact of sporulation temperature on the germination kinetics of *Bacillus subtilis* spores under optimal and adverse temperature, pH, or a_w conditions used for food preservation, and to compare their minimal boundaries for the inhibition of germination. *B. subtilis* is often involved in food spoilage, and some strains are toxigenic and have been occasionally involved in food poisoning incidents (André et al., 2017; Apetroaie-Constantin et al., 2009). Our study covered spore response to a variety of nutrient stimuli using the same experimental setup, and the chosen range of sporulation temperatures equally encompassed stressful low and high values in terms of sporulation efficiency (Freire et al., 2023). This should allow us to establish meaningful comparisons and enable researchers to elucidate discrepancies previously reported in the literature.

2. Material and methods

2.1. Obtention and purification 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 250-mL flasks containing 20 mL of liquid 2 × SG sporulation medium (Freire et al., 2023). Flasks were incubated at 20 °C, 37 °C, and 43 °C with shaking (130 rpm) until two equal spore counts, determined by plating aliquots previously exposed to a thermal treatment (75 °C, 15 min), were obtained on two successive days (7 days at 20 °C, 4 days at 37 °C, and 4 days at 43 °C; Freire et al., 2023). Spores were harvested by centrifugation at 3345 *g* for 20 min at 4 °C and washed 3 times with sterile distilled water. They were then purified by buoyant density centrifugation using Nycodenz®, as described in a former study (Ghosh & Setlow, 2009). Spore purity (98 % bright spores) was verified by phase contrast microscopy (Nikon Eclipse E400, Tokyo, Japan), and the suspensions were kept at −20 °C until use. To assess biological variability, three different spore populations were obtained on independent working days at each temperature.

2.2. Nutrient-induced germination assays

Germination assays were performed in Nutrient Broth No. 2 supplemented with 0.6 % of yeast extract (NBYE) as a rich growth medium, or in 25 mM HEPES buffer with a saturating concentration (10 mM) of L-alanine (Sigma-Aldrich, St. Louis, MO, USA), L-valine (Sigma-Aldrich), or AGFK mixture (L-asparagine [Amresco Inc., Solon, Ohio, USA], D-glucose [Panreac, Barcelona, Spain], D-fructose [Panreac], and KCl [Panreac]) (Butzin et al., 2012; Yi & Setlow, 2010; Zhang et al., 2014). NBYE was supplemented with 4 mg/mL of ampicillin (Sigma-Aldrich) to avoid the interference of growth of the first germinated spores (Paidhungat & Setlow, 2000). The antibiotic's presence did not affect germination behaviour (data not shown). To evaluate germination under adverse preservation conditions, pH of the germination medium was acidified (from pH 7.0 to 5.0) by adding HCl 1 M (Panreac), and water activity was reduced (from $a_w > 0.99$ to 0.96) by adding NaCl (Panreac), sucrose (Panreac), or glycerol (Panreac). pH was adjusted 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). All experiments were carried out at 37 °C, excepting a few performed at 20 °C to test the impact of a lower incubation temperature.

Spores were suspended in each germination medium at a final optical density at 600 nm (OD_{600}) of 0.4–0.6. To test the effect of heat activation, spores were treated at 65 °C for 30 min in a PCR machine (Bio-Rad, T100 Thermocycler, Hercules, CA, USA) prior to nutrient exposure. Preliminary experiments had shown that these treatment conditions were best at improving the responsiveness of all spore populations to all the nutrients. Germination kinetics were followed by OD_{600} drop in a multiwell plate reader (CLARIOstar Plus, BMG, Ortenberg, Germany) that automatically measured values each 3 min for 3–4 h while shaking 30 s between reads to avoid spore sedimentation. When germination was tested at 20 °C, we extended the incubation time to 24 h to ensure that the plateau phase was reached. Germination curves were constructed using the percentage of OD_{600} fall ($OD_t/OD_i \times 100$, where OD_i and OD_t represent the initial value and the value measured at further incubation times, respectively).When indicated, germination kinetics were also monitored by DPA-Tb fluorescence, supplementing each sample with a final concentration of 50 μM $TbCl_3$ (Sigma-Aldrich). Fluorescence was automatically measured with the above indicated multiwell plate reader, with similar kinetics parameters and using an excitation wavelength of 270 nm and an emission wavelength of 545 nm (Yi & Setlow, 2010). Curves obtained by DPA-Tb fluorecence were constructed using the percentage of DPA release ($F_t/F_i \times 100$, where F_i and F_t represent the fluorescence of an autoclaved sample and the one measured at different incubation times, respectively).

After each spectrophotometry or DPA-Tb fluorometry assay, we determined the percentage of germinated spores by phase contrast microscopy. A number of 100 to 150 individuals per sample was examined and counted either as dormant (phase bright) or germinated spores (phase dark and grey). For each nutrient and environmental condition, we obtained three germination curves from different biological replicates.

2.3. Chemical-induced germination assays

For Ca-DPA-induced germination, spores (OD_{600} 0.4–0.6) were incubated in 25 mM HEPES buffer of pH 7.0 with DPA (Sigma-Aldrich) and $CaCl_2$ (VWR, Radnor, Pennsylvania, USA), both added to a final concentration of 50 mM. For dodecylamine-induced germination, spores (OD_{600} 0.4–0.6) were incubated 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). Germination kinetics in CaDPa was evaluated by spectrophotometry at 37 °C (Setlow et al., 2009), while germination kinetics in dodecylamine was monitored by DPA-Tb fluorescence at 45 °C (DeMarco et al., 2021) as described above. The percentage of germinated spores at the end of the assays was examined by phase contrast microscopy. For each chemical, we obtained three germination kinetics from different biological replicates.

2.4. Modelling of germination curves

Germination curves obtained by OD_{600} measurements were fit to the One-Phase Decay equation (Eq. 1) using GraphPad PRISM 5.0 (GraphPad Software Inc., San Diego, CA, USA). This model describes germination curves with two parameters: k (min^{-1}), which represents the germination rate constant, and *plateau*, which indicates the percentage of OD_{600} fall at infinite times. When a lag phase was evident prior to the exponential decay, the Plateau followed by One-Phase Decay model (Eq. 2) was used instead: this model incorporates the *lag* parameter indicating the duration of such a phase. To assess goodness of fit, we calculated the R^2 and root mean square error (RMSE).

$$OD_{600}\ \text{fall}\ (\%) = (100 - plateau)\ e^{(-kt)} + plateau \quad (1)$$

$$OD_{600}\ \text{fall}\ (\%) = (100 - plateau)\ e^{(-k(t-lag))} + plateau \quad (2)$$

Curves obtained under environmental conditions in which OD_{600} fell to less than 15 % were not modelled. To enable a more precise comparison among various germination conditions, the study employed microscopic observation as a means of assessing germination efficiency.

2.5. Germination boundary assays

Temperature, pH, and a_w boundaries for germination were determined in NBYE (Oxoid) supplemented with ampicillin (4 mg/mL). Each environmental variable was studied individually while maintaining the other ones at optimal values (37 °C, pH 7.0, and $a_w > 0.99$). Samples with an initial spore concentration of ca. 1×10^8 spores/mL were incubated up to 7 days at temperatures ranging from 8 °C to 16 °C (2 °C increments), 4 days at pH between 5.0 and 3.0 (0.2 increments), or 4 days at a_w between 0.97 and 0.94 with NaCl, between 0.96 and 0.88 with glycerol, or between 0.96 and 0.85 with sucrose (0.1 increments). Longer incubation times did not significantly improve germination efficiency. The percentage of germinated spores was determined by examining ca. 100 spores under phase contrast microscopy. Germination boundaries were set to temperature, pH, and a_w values that decreased germination by 90 % or more compared to the proportion of phase dark spores in the sample before incubation (<2 %).

2.6. Statistical analysis

Using GraphPad PRISM 5.0, we performed one-way ANOVA with Tukey's post hoc. When comparing two conditions, we performed the unpaired parametric *t*-test. Differences were considered statistically significant when P was ≤ 0.05. Data in the figures correspond to averages and standard deviations calculated from three biological replicates obtained on different days.

3. Results and discussion

3.1. Germination triggered by nutrients under optimal conditions

We first examined the germination kinetics of spores produced at 37 °C, 20 °C, and 43 °C (designated as $S_{37°C}$ or control spores, $S_{20°C}$, and $S_{43°C}$, respectively) in NBYE as a rich growth medium and in the well-known germinants L-alanine, L-valine, and AGFK under optimal conditions (pH 7.0, 37.0 °C, $a_w > 0.99$) by spectrophotometry (Fig. 1). When possible, we also evaluated germination by DPA-Tb fluorescence

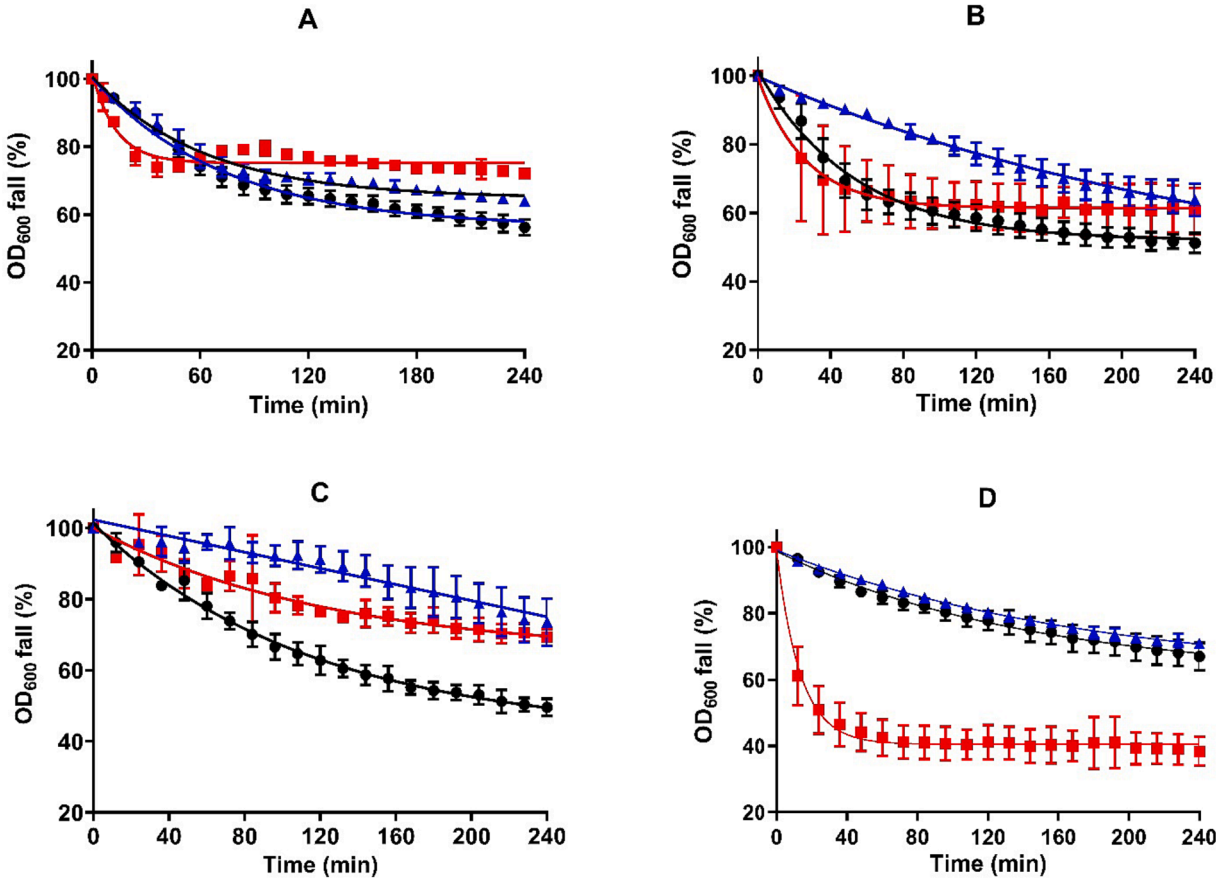


Fig. 1. Germination curves of $S_{20°C}$ (blue, triangles), $S_{37°C}$ (black, circles), and $S_{43°C}$ (red, squares) spores in NBYE (pH 7.0, $a_w > 0.99$; A) and HEPES buffer (pH 7.0, $a_w > 0.99$) with 10 mM L-alanine (B), L-valine (C), or AGFK (D) at 37 °C. Germination represents the percentage of OD_{600} fall ($OD_t/OD_0 \times 100$) over time. Data in the figures correspond to averages and standard deviations calculated from three biological replicates.

(Fig. S1). Table 1 shows the germination rate (k) obtained after fitting germination curves to the exponential decay model (Eq. 1), together with the percentage of DPA release and the percentage of phase dark spores at the end of the 4-hour assay. Control spores showed a higher ($P \leq 0.05$) germination rate and efficiency in NBYE and L-alanine than in L-valine. Induction of germination of these spores with AGFK resulted in even lower ($P \leq 0.05$) k values and a lower percentage of germinated spores than with L-valine.

The influence of sporulation temperature on germination was more pronounced in the three specific germinants than in NBYE, and the effect's magnitude and direction depended on the type of nutrient. Spores obtained at 20 °C displayed a lower germination rate and efficiency in most nutrients than their counterparts produced at the optimal temperature. The germination rate in L-alanine decreased ($P \leq 0.05$) 6.5-fold when sporulation temperature was reduced from 37 °C to 20 °C (Fig. 1B, Table 1). After 4 h of incubation, germination was triggered in 89.8 % of $S_{37°C}$ spores, while only 41.3 % of $S_{20°C}$ spores turned to phase dark (Table 1). In the same vein, $S_{20°C}$ spores hardly responded at all to L-valine, showing 5.0-fold and 7.1-fold lower ($P \leq 0.05$) germination efficiency and total DPA release than $S_{37°C}$ spores (Fig. 1C, Table 1). Interestingly, $S_{20°C}$ spores germinated just as slowly as ($P > 0.05$) $S_{37°C}$ spores in AGFK, although the final percentage of germinated spores and DPA release was slightly higher ($P \leq 0.05$) in those which had sporulated at the optimal temperature (Fig. 1D, Table 1).

Raising sporulation temperature over the optimum improved the rate of germination in NBYE, L-alanine, and AGFK. This effect was particularly marked in AGFK, where k values of $S_{43°C}$ spores were 12.6-fold higher ($P \leq 0.05$) than the k values of those sporulated at the optimal temperature (Fig. 1D, Table 1). In keeping with those results, AGFK induced germination in 95.6 % of $S_{43°C}$ spores, while only 49.5 % of $S_{37°C}$ spores germinated (Table 1). Thus, $S_{43°C}$ spores germinated in this mixture just as well as in L-alanine, which was the most efficient germinant in the control population. In contrast, an increase in sporulation temperature reduced ($P \leq 0.05$) germination efficiency and total DPA released in L-valine (1.2-fold and 1.4-fold, respectively) but to a lesser degree than decreasing sporulation temperature to 20 °C (5.0-fold and 7.1-fold, respectively; Fig. 1C, Table 1).

We also compared the effect of sporulation temperature on germination in NBYE, L-alanine, L-valine, and AGFK after the spores had been exposed to a heat treatment of 65 °C for 30 min (Fig. S2, Table S1). Such treatment increased ($P \leq 0.05$) k values of control spores in NBYE, in L-alanine, and, especially, in AGFK, where it also induced a 1.7-fold increase in germination efficiency. As previously noted by Luu et al. (2015), spores produced at suboptimal temperature experienced a greater increase in germination rate and/or efficiency by heat activation than $S_{37°C}$ spores in any of the nutrients. Indeed, germination speed and percentage of germination in $S_{20°C}$ spores surpassed those values for $S_{37°C}$ spores in AGFK, although in L-alanine and L-valine they remained the least fit populations. In contrast, heat activation barely benefitted, and occasionally even impaired germination of $S_{43°C}$ spores. Heat activation thus attenuated differences in germination caused by variations in sporulation temperature, so that the advantages of $S_{43°C}$ spores in terms of germination rate and/or efficiency diminished (in AGFK and NBYE) and even disappeared (in L-alanine) with regard to $S_{37°C}$ spores. These outcomes suggest that contrary results found in the literature may be partly explained by the application of a heat activation treatment and perhaps by differences in the intensity applied.

Although several studies have addressed the impact of sporulation temperature on spore germination, the nature and regulation of changes in spore structure and composition leading to different behaviours is still poorly understood. This is further obscured by the contradictory data reported in the literature, even using similar experimental setups (Gounina-Allouane et al., 2008; Luu et al., 2015; Ramirez-Peralta et al., 2012). Ramirez-Peralta et al. (2012) concluded that the lower germination rate and efficiency of *B. subtilis* PS832 spores produced at 23 °C in L-valine and AGFK compared to those incubated at 37 °C could be partly attributed to lower germinant receptor (GR) levels of the former. If this had occurred in our case, changes in sporulation temperature would have exerted different effects on the expression of GerA, GerB, and GerK, given that a reduction of sporulation temperature below the optimum impaired germination more efficiently in L-alanine and L-valine than in AGFK. Moreover, $S_{43°C}$ spores in L-alanine showed a significantly higher germination rate but similar germination efficiency than $S_{37°C}$ spores in the same medium, while an equal k value but a lower percentage of germination in $S_{43°C}$ spores could be observed in L-valine, despite the fact that the two amino acids act on the same type of GR (Moir & Smith, 1990). All these findings suggest that other factors, apart from varying levels of GR, may contribute to the better capacity of $S_{43°C}$ spores to germinate in L-alanine, in AGFK, and in NBYE.

Table 1
Germination rate obtained after fitting germination curves to the exponential decay model (Eq. 1), and the percentage of germinated spores and DPA released at the end of the assay (4 h) in different nutrient germinants under optimal conditions (37 °C, pH 7.0, $a_w > 0.99$) (Fig. 1). Values in brackets correspond to standard deviations of the means calculated from three biological replicates. Data were statistically analysed using one-way ANOVA test with Tukey's post hoc. When only two sporulation temperatures were compared, the unpaired parametric t-test was applied. Different lowercase letters indicate statistically significant differences ($P \leq 0.05$) among spore populations produced at different sporulation temperatures within each germinant, while different capital letters indicate statistically significant differences ($P \leq 0.05$) among germinants within each population sporulated at a specific temperature.

Germinant	Population	k (min^{-1})	R^2	RMSE	Germination efficiency (%)	DPA released (%)
NBYE	$S_{37°C}$	0.0171^{aAB} (0.0031)	0.990	1.20	91.28^{aA} (2.55)	N.D.
	$S_{20°C}$	0.0140^{aA} (0.0016)	0.983	1.23	70.13^{bA} (4.65)	N.D.
	$S_{43°C}$	0.0591^{bA} (0.0032)	0.973	1.11	89.58^{aA} (3.50)	N.D.
L-alanine	$S_{37°C}$	0.0203^{aA} (0.0038)	0.994	0.92	89.80^{aA} (4.51)	87.64^{aA} (6.69)
	$S_{20°C}$	0.0031^{bB} (0.0009)	0.983	1.43	41.25^{bB} (10.33)	30.63^{bA} (10.01)
	$S_{43°C}$	0.0735^{cAC} (0.0095)	0.959	2.31	82.85^{aAB} (11.47)	81.05^{aA} (2.57)
L-valine	$S_{37°C}$	0.0120^{aBC} (0.0030)	0.991	1.22	79.98^{aB} (2.63)	69.07^{aB} (5.94)
	$S_{20°C}$	N.M.	N.M.	N.M.	15.84^{bC} (2.92)	9.68^{bB} (7.10)
	$S_{43°C}$	0.0140^{aB} (0.0050)	0.951	2.19	65.36^{cB} (6.47)	49.67^{cB} (8.62)
AGFK	$S_{37°C}$	0.0065^{aC} (0.0022)	0.996	0.51	49.46^{aC} (4.76)	30.26^{aC} (2.78)
	$S_{20°C}$	0.0051^{aB} (0.0012)	0.988	0.94	26.69^{bBC} (6.40)	14.33^{bAB} (5.32)
	$S_{43°C}$	0.0820^{bC} (0.0160)	0.961	1.92	95.56^{cA} (2.95)	88.50^{cC} (0.19)

N.D.: not determined. The percentage of DPA released could not be determined in NBYE because its components interfere with DPA-Tb chelation.
N.M.: Not modelled. OD_{600} values did not decrease >15 %.

3.2. Germination triggered by non-nutrient germinants

In order to obtain further mechanistic insights into the varying responses to nutrients observed in populations sporulated at different temperatures, we studied germination kinetics with Ca-DPA, which induces germination by activating the cortex lytic enzyme CwlJ (Paidhungat, Ragkousi, & Setlow, 2001), and with dodecylamine, which is believed to disrupt the inner membrane, leading to release of DPA (Mokashi et al., 2020). All Ca-DPA germination curves displayed a lag phase, which was significantly ($P \leq 0.05$) shorter in spores produced at the suboptimal temperature (15.1 min) than in $S_{37°C}$ (22.5 min) and $S_{43°C}$ (26.5 min) spores (Fig. 2A; Table 2). In addition, $S_{20°C}$ spores had a higher ($P \leq 0.05$) germination rate and germination efficiency at the end of the assay (4 h) in comparison to those sporulated at the optimal temperature and at the highest temperature (Table 2). When exposed to dodecylamine, the rate of DPA release was higher in $S_{20°C}$ spores, followed by $S_{43°C}$ and $S_{37°C}$ spores (Fig. 2B). This finding agrees with the fact that $S_{20°C}$ and, to a lesser extent, $S_{43°C}$ populations are more sensitive to an inactivation treatment using dodecylamine (Freire et al., 2023). Ramirez-Peralta et al. (2012) also observed that spores produced at suboptimal temperature displayed higher dodecylamine germination efficiency than those sporulated at the optimal temperature, although both populations germinated in Ca-DPA to a similar extent.

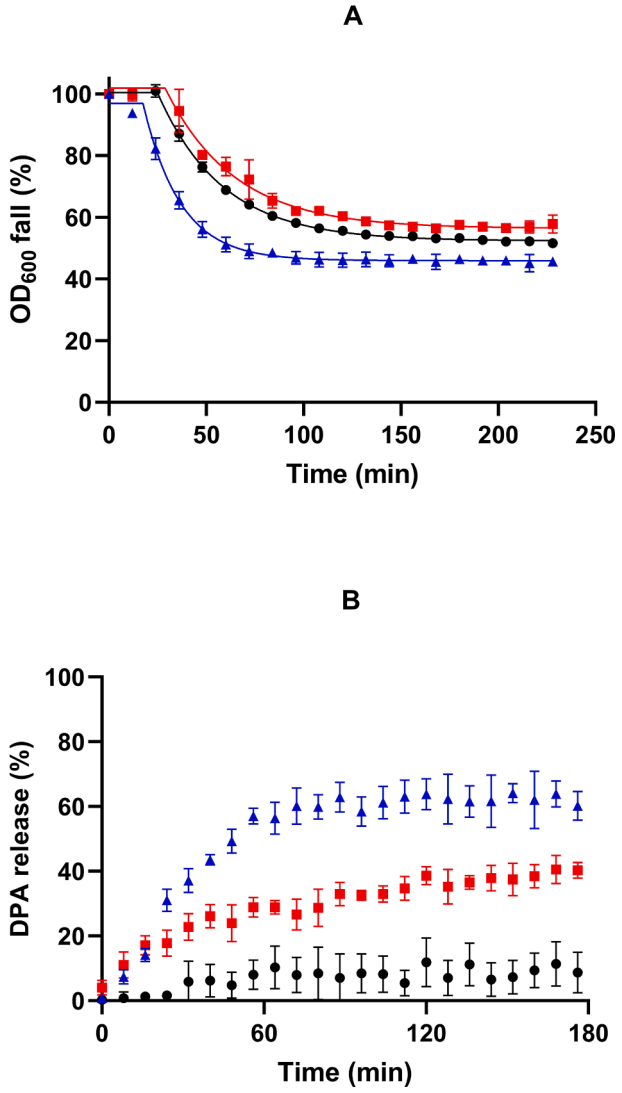


Fig. 2. Germination curves of $S_{20°C}$ (blue, triangles), $S_{37°C}$ (black, circles), and $S_{43°C}$ (red, squares) spores in Ca-DPA (50 mM, pH 7.0, 37 °C; A) and in dodecylamine (25 mM, pH 9.0, 45 °C; B). Germination in Ca-DPA represents the percentage of OD_{600} fall, while germination in dodecylamine corresponds to the percentage of DPA-Tb fluorescence increase. Data in the figures correspond to averages and standard deviations calculated from three biological replicates.

Table 2
Germination kinetics parameters (*lag* and *k* values) obtained after fitting germination curves to the lag + exponential decay model (Eq. 2) and the percentage of germinated spores at the end of the assay (4 h) in Ca-DPA (Fig. 2A). Values in brackets correspond to standard deviations of the means calculated from three biological replicates. Data were statistically analysed using one-way ANOVA test with Tukey's post hoc. Different lowercase letters indicate statistically significant differences ($P \leq 0.05$) among spore populations produced at different sporulation temperatures.

Population	*lag* (min)	*k* (min^{-1})	R^2	RMSE	Germination efficiency (%)
$S_{37°C}$	22.47[a] (2.01)	0.0300[a] (0.0025)	0.999	1.01	86.89[a] (2.17)
$S_{20°C}$	15.08[b] (2.40)	0.0460[b] (0.0013)	0.998	1.03	98.10[b] (1.68)
$S_{43°C}$	26.54[c] (0.69)	0.0270[a] (0.0008)	0.981	2.17	92.30[ab] (3.73)

The fact that spores produced at the lowest temperature responded better to Ca-DPA than spores produced at 37 °C might indicate that $S_{20°C}$ and $S_{37°C}$ spores have different levels of CwlJ and/or a different degree of permeability to Ca-DPA. The supposition that $S_{20\ °C}$ spores possess a higher degree of permeability is supported by the fact that spores produced at suboptimal temperature are more sensitive to certain chemicals acting on the inner membrane, such as sodium hypochlorite and dodecylamine (Fig. 2B; Freire et al., 2023), as well as to core components, such as DNA-damaging agents (Cortezzo & Setlow, 2005). At any rate, degradation of the cortex does not seem to be the step that impairs the germination of spores produced at the lowest sporulation temperature. Several studies have suggested that changes in coat composition and structure could play a significant role in the varying nutrient permeability of spores incubated at different temperatures (Bressuire-Isoard et al., 2016; Ghosh et al., 2008; Isticato et al., 2020) . For instance, Saggese et al. (2022) recently reported that a *B. subtilis* PY79 mutant lacking the *cotG* gene or its internal repeats, which encodes a coat protein that was more abundantly extracted from the rapidly germinating spores produced at 25 °C than from the more slowly germinating spores obtained at 42 °C (Di Gregorio Barletta et al., 2022; Isticato et al., 2020), displayed germination defects in AGFK. Further research is needed to fully understand which changes in spore structures and properties induced by variations in sporulation temperature are responsible for differences in spore behaviour.

3.3. Nutrient germination under adverse conditions

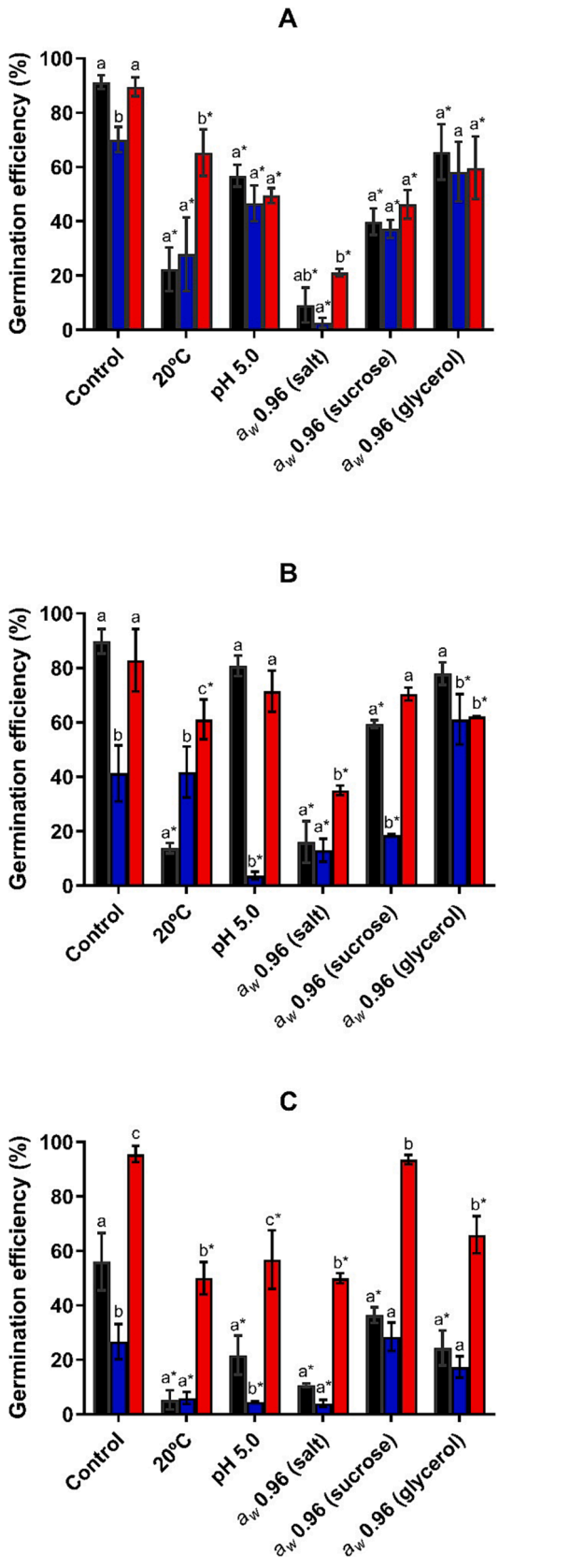
Since control of spore germination in the storage of food products that are not subjected to an intensive sterilization treatment relies on a series of hurdles, including refrigeration, acidification, and/or the addition of solutes, we assessed whether differences in germination observed among spores produced at 20 °C, 37 °C, and 43 °C under optimal environmental conditions could also be observed under food-related stresses. More specifically, we examined the germination rate and efficiency of the three populations under conditions of temperature, pH, and a_w that impaired germination of spores obtained at the optimal temperature but still permitted the growth of resulting vegetative cells (Table 3, Fig. 3). Germination was tested only in NBYE, L-alanine, and AGFK, where non-activated $S_{43°C}$ spores displayed better fitness in comparison to those of $S_{37°C}$ and $S_{20°C}$.

We observed that germination speed and germination efficiency

Table 3
Germination rate obtained after fitting germination curves in NBYE, L-alanine, and AGFK, changing temperature (to 20 °C), pH (to 5.0) or a_w (to 0.96) with different solutes (NaCl, glycerol, or sucrose) from the optimum (control; 37 °C, pH 7.0 $a_w > 0.99$), to the exponential decay models (Eqs. (1) and (2). Values in brackets correspond to standard deviations of the means calculated from three biological replicates. Data were statistically analysed using one-way ANOVA test with Tukey's post hoc. When only two sporulation temperatures were compared, the unpaired *t*-test was applied. Different lowercase letters indicate statistically significant differences ($P \leq 0.05$) among spore populations under each environmental condition within each germinant. An asterisk indicates statistically significant differences ($P \leq 0.05$) between control conditions and those changed to an adverse temperature, pH, or a_w within each germinant and spore population.

	Condition changed	Population	k (min^{-1})	R^2	RMSE		Condition changed	Population	k (min^{-1})	R^2	RMSE		Condition changed	Population	k (min^{-1})	R^2	RMSE
NBYE	Control	$S_{37°C}$	0.0171^{a} (0.0031)	0.990	1.23	L-alanine	Control	$S_{37°C}$	0.0203^{a} (0.0038)	0.994	0.93	AGFK	Control	$S_{37°C}$	0.0065^{a} (0.0022)	0.996	0.52
		$S_{20°C}$	0.0140^{a} (0.0016)	0.983	1.20			$S_{20°C}$	0.0031^{b} (0.0009)	0.983	1.42			$S_{20°C}$	0.0051^{a} (0.0012)	0.988	0.93
		$S_{43°C}$	0.0591^{b} (0.0032)	0.973	1.19			$S_{43°C}$	0.0735^{c} (0.0095)	0.959	2.01			$S_{43°C}$	0.0820^{b} (0.0161)	0.961	1.91
	20 °C	$S_{37°C}$	0.0020^{a}* (0.001)	0.960	1.06		20 °C	$S_{37°C}$	0.0120^{a}* (0.0029)	0.920	1.62		20 °C	$S_{37°C}$	0.0106^{a} (0.0024)	0.880	1.60
		$S_{20°C}$	0.0017^{a}* (0.0003)	0.980	0.87			$S_{20°C}$	0.0077^{a} (0.0029)	0.962	1.53			$S_{20°C}$	0.0045^{b} (0.0028)	0.804	2.22
		$S_{43°C}$	0.0320^{b}* (0.0061)	0.927	0.94			$S_{43°C}$	0.0395^{b} (0.0243)	0.949	1.32			$S_{43°C}$	0.0545^{c} (0.0295)	0.900	0.018
	pH 5.0	$S_{37°C}$	0.0301^{a}* (0.002)	0.967	1.25		pH 5.0	$S_{37°C}$	0.0203^{a} (0.0026)	0.962	1.56		pH 5.0	$S_{37°C}$	0.0121^{a} (0.0049)	0.950	0.82
		$S_{20°C}$	0.0120^{b} (0.0011)	0.993	0.75			$S_{20°C}$	0.0006^{b}* (0.0001)	0.986	1.49			$S_{20°C}$	N.M.	N.M.	N.M.
		$S_{43°C}$	0.1484^{c}* (0.0479)	0.974	0.96			$S_{43°C}$	0.0509^{c} (0.0128)	0.988	1.26			$S_{43°C}$	0.0391^{b}* (0.0128)	0.953	1.35
	a_w 0.96 - NaCl	$S_{37°C}$	N.M.	N.M.	N.M.		a_w 0.96 - NaCl	$S_{37°C}$	N.M.	N.M.	N.M.		a_w 0.96 - NaCl	$S_{37°C}$	N.M.	N.M.	N.M.
		$S_{20°C}$	N.M.	N.M.	N.M.			$S_{20°C}$	N.M.	N.M.	N.M.			$S_{20°C}$	N.M.	N.M.	N.M.
		$S_{43°C}$	0.1310* (0.0267)	0.955	1.43			$S_{43°C}$	0.0914 (0.0301)	0.956	1.58			$S_{43°C}$	0.1261 (0.0540)	0.958	1.83
	a_w 0.96 - sucrose	$S_{37°C}$	0.0279^{a}* (0.0047)	0.998	1.06		a_w 0.96 - sucrose	$S_{37°C}$	0.0367^{a}* (0.0038)	0.990	1.32		a_w 0.96 - sucrose	$S_{37°C}$	0.0149^{a}* (0.0041)	0.983	0.99
		$S_{20°C}$	0.0248^{a}* (0.0053)	0.990	1.17			$S_{20°C}$	0.0109^{b}* (0.0025)	0.995	1.12			$S_{20°C}$	0.0015^{b}* (0.0001)	0.990	0.90
		$S_{43°C}$	0.1100^{b}* (0.0247)	0.983	3.13			$S_{43°C}$	0.0901^{c}* (0.0118)	0.990	1.80			$S_{43°C}$	0.0483^{c}* (0.0082)	0.986	2.29
	a_w 0.96 - glycerol	$S_{37°C}$	0.0230^{a} (0.0036)	0.982	1.01		a_w 0.96 - glycerol	$S_{37°C}$	0.0221^{a} (0.0014)	0.983	0.97		a_w 0.96 - glycerol	$S_{37°C}$	0.0059^{a} (0.0022)	0.954	1.04
		$S_{20°C}$	0.0162^{a} (0.0029)	0.957	0.87			$S_{20°C}$	N.M.	N.M.	N.M.			$S_{20°C}$	0.0016^{b}* (0.0001)	0.939	0.84
		$S_{43°C}$	0.0558^{b} (0.0081)	0.985	1.76			$S_{43°C}$	0.0526^{b}* (0.0068)	0.960	1.38			$S_{43°C}$	0.0426^{c}* (0.0077)	0.958	1.83

N.M.: not modelled. OD_{600} values did not decrease more than 15 %.



(caption on next column)

Fig. 3. Germination efficiency of $S_{37°C}$ (black, first column), $S_{20°C}$ (blue, second column), and $S_{43°C}$ (red, third column) spores in NBYE (A) or HEPES buffer with 10 mM L-alanine (B) or AGFK (C), changing temperature (to 20 °C), pH (to 5.0), or a_w (to 0.96) with different solutes (NaCl, glycerol, or sucrose) from the optimum (37 °C, pH 7.0, $a_w > 0.99$). The percentage of germinated spores was determined microscopically after 4 h of incubation at 37 °C, and this time was extended to 24 h when incubated at 20 °C. Values in the figures correspond to averages and standard deviations calculated from three biological replicates. Data were statistically analysed using one-way ANOVA test with Tukey's post hoc. Different lowercase letters indicate statistically significant differences ($P \leq 0.05$) among spore populations produced at different sporulation temperatures under each environmental condition within each germinant. Asterisk indicates statistically significant differences ($P \leq 0.05$) between each adverse environmental condition and the optimal one within each population.

decreased at a varying rate when spores were incubated at 20 °C compared to 37 °C, depending on the nutrient and on sporulation temperature. The lowering of incubation temperature decreased ($P \leq 0.05$) the germination rate of control spores almost 10-fold in NBYE, whereas it only changed ($P \leq 0.05$) 1.7-fold in L-alanine and remained invariable ($P > 0.05$) in AGFK (Table 3). The fraction of $S_{37°C}$ spores able to germinate after reaching the plateau phase (4 h at 37 °C and 24 h at 20 °C) was reduced ca. 5.3-fold in both NBYE and L-alanine and 10.5-fold in AGFK (Fig. 3). Germination rate and germination efficiency of $S_{43°C}$ spores were generally less impaired by the reduction of incubation temperature than was the case for $S_{37°C}$ and $S_{20°C}$ spores; the former thus maintained their superior germination fitness, thereby enlarging differences among populations induced by sporulation temperature. For instance, k values and the proportion of phase dark spores of $S_{43°C}$ populations in NBYE only decreased ($P \leq 0.05$) by less than half as a consequence of the change in germination temperature (Table 3, Fig. 3). An exception could be observed in the $S_{20°C}$ spores exposed to L-alanine, which behaved equally at 37 °C and 20 °C; moreover, at 20 °C, they displayed higher ($P \leq 0.05$) germination efficiency than $S_{37°C}$ populations, although still lower ($P \leq 0.05$) than $S_{43°C}$ spores (Fig. 3). Along similar lines, Garcia et al., (2010) reported that *B. weihenstephanensis* spores formed at the highest temperature (30 °C) germinated more efficiently in a mixture of inosine and L-alanine at optimal (30 °C) and suboptimal growth temperatures (20 °C and 12 °C). In single-cell approaches, it has been reported that reducing germination temperature extends the lag phase and the bright-to-dark transition phase; this has been attributed to the effect of temperature changes on nutrient permeability and the activity of the enzymes involved (Billon et al., 1997; Leuschner & Lillford, 1999; Stringer et al., 2009; Trunet et al., 2020) Our results therefore suggest that sporulation temperature may exert an influence on spore properties that are important for the accessibility of certain nutrients and/or the activity of the preformed enzymes that are susceptible to lower germination temperatures.

Acidification has been shown to delay germination and reduce the fraction of spores capable of germinating in a specific pH range for each strain and nutrient (Broussolle et al., 2008; Ciarciaglini et al., 2000; Trunet et al., 2020). Our study indicates that, in *B. subtilis*, this also depends on the sporulation temperature. Reducing pH from 7.0 to 5.0 decreased ($P \leq 0.05$) germination efficiency of $S_{37°C}$, $S_{20°C}$, and $S_{43°C}$ populations in NBYE to the same level ($P > 0.05$; Fig. 3), although $S_{43°C}$ populations still had the highest germination rate (Table 3). In L-alanine, acidification did not affect ($P > 0.05$) germination of $S_{37°C}$ and $S_{43°C}$ spores but it decreased ($P \leq 0.05$) k values and the proportion of germinated spores of $S_{20°C}$ populations 5.2- and 11.2-fold, respectively (Table 3, Fig. 3). Germination of $S_{20°C}$ spores in AGFK was almost entirely inhibited under acidic conditions (Table 3, Fig. 3). Moreover, although acidification decreased ($P \leq 0.05$) k values of $S_{43°C}$ spores by a 2.1-fold rate, germination efficiency was more reduced in $S_{20°C}$ (5.9-fold) than in $S_{37°C}$ (2.6-fold) and $S_{43°C}$ populations (1.7-fold) (Table 3, Fig. 3). Blocher & Busta (1985)suggested that inhibition of amino acid-induced germination of *B. cereus* and *C. botulinum* spores at

low pH was due to the obstruction of ligand-GR binding by protonation of a functional group in or near the trigger site. Our results thus suggest that such an effect might depend on the type of nutrient-GR binding, as well as on other features of the surrounding environment that can be modified by altering the sporulation temperature. Overall, variations in germination kinetics induced by sporulation temperature observed at pH 7.0 became more pronounced at pH 5.0, especially in L-alanine. Moreover, despite the constraint imposed by acidification, $S_{43°C}$ spores still displayed a more advantageous germination rate and/or efficiency with regard to control spores.

We studied the impact of reducing a_w to 0.96 using either NaCl, glycerol, or sucrose as a solute. As expected (Anagnostopoulos & Sidhu, 1981; Nagler et al., 2014; Rao et al., 2018), lowering a_w impaired germination efficiency to a varying extent depending on the nutrient and the solute used (Fig. 3). NaCl was the most effective a_w depressor, reducing to ≤16 % the fraction of control spores able to germinate after 4-hour exposure to all the types of nutrients under study. Adding sucrose decreased ($P \leq 0.05$) germination efficiency ca. 2.3-fold only in NBYE and AGFK, while glycerol reduced ($P \leq 0.05$) it to a lesser extent (ca. 1.5-fold) in the three nutrients. However, as opposed to previously reported results (Nagler et al., 2014; Rao et al., 2018, we did not observe a decrease of *k* values in the presence of any of the three solutes (Table 3). Further microscopic analysis is required to clarify this discrepancy.

The effect of depressing a_w on germination efficiency also depended on the sporulation temperature (Fig. 3). Adding NaCl reduced the proportion of germinated spores to a lower extent in $S_{43°C}$ than in $S_{37°C}$ and $S_{20°C}$ populations, especially in the rich medium. While the presence of NaCl decreased ($P \leq 0.05$) the percentage of germination in $S_{37°C}$ populations 10.0-fold in NBYE and ca. 5.4-fold in L-alanine and AGFK, it only reduced that fraction in $S_{43°C}$ spores 4.2-, 2.4-, and 1.9-fold, respectively. Moreover, although no differences ($P > 0.05$) in germination efficiency were observed between $S_{37°C}$ and $S_{43°C}$ spores in L-alanine at optimal a_w, the percentage of phase dark spores in $S_{43°C}$ populations at the end of the assay was twice ($P \leq 0.05$) that of $S_{37°C}$ spores in the presence of NaCl. Both sucrose and glycerol diminished the percentage of germination to the same level in NBYE independently of the sporulation temperature. However, depressing a_w with glycerol affected germination efficiency of $S_{43°C}$ spores in L-alanine and AGFK to a lesser extent than in the control spores. The presence of sucrose in AGFK buffer hardly ($P > 0.05$) depressed the fraction of germinated spores in $S_{43°C}$ and in $S_{20°C}$ populations at all; thus, $S_{43°C}$ spores remained the ones which germinated best under that condition. Therefore, germination of populations produced at the highest temperature suffered less from a_w reduction and these spores displayed the best fitness in most scenarios (except when adding sucrose or glycerol in NBYE and L-alanine), while differences in germination efficiency among the three populations increased.

Nagler et al. (2014) observed that the required time for initiating germination and for releasing the great majority of spores' DPA increased in individual *B. subtilis* spores exposed to nutrients with a NaCl concentration similar to the concentration applied in our study (ca. 1.2 M). Although it has been suggested that NaCl may impair ligand-GR binding, nutrient accessibility, ion and DPA release, and/or water uptake, its actual inhibitory mechanism remains a mystery (Nagler & Moeller, 2015; Nagler et al., 2014; Nagler et al, 2015) On the other hand, Rao et al. (2018) reported that reducing a_w (0.97–0.90) with either glycerol or sucrose mostly affected the amount of time necessary for commitment as well as the duration of the initially slow DPA release in sucrose. The same authors suggested that it is more plausible that the different degree of hydration of GR rather than the solute effect on germinant penetration is involved in the delay of germination onset. Our results indicate that $S_{43°C}$ spores possess a series of differentiating features that make them the most advantageous spores for germination at low a_w in a wide variety of nutrients and solutes. One of the candidates for the explanation of this phenomenon is the coat, which has shown varying structure and composition in spores produced at different temperatures (Melly et al., 2002; Isticato et al., 2020) and plays a protective role when germinating at reduced a_w (Nagler et al., 2015; Rao et al., 2018). Nevertheless, the fact that $S_{43°C}$ populations responded differently to each nutrient when depressing a_w with the same solute suggests that other factors may likewise be involved.

3.4. Impact of sporulation temperature on temperature, pH, and a_w boundaries for germination

The varying effect of adverse temperature, pH, and a_w with different solutes on the germination of spores produced at 20 °C, 37 °C, and 43 °C incited us to explore whether germination boundaries also changed along with sporulation temperature. Indeed, it is important to have knowledge of boundary preservation conditions for the inhibition of germination, as only a reduced number of spores capable of germinating can already cause food stability or safety issues. To this end, we preferred NBYE over single germinants due to its rich composition, and we set the lowest threshold for germination at conditions at which the percentage of germinated spores examined under the microscope did not surpass 10 % (Table 4).

$S_{43°C}$ spores showed a >6 °C lower temperature limit for germination than those that had sporulated at the optimal and suboptimal temperature. At 8 °C, $S_{43°C}$ spores were able to germinate up to 12.6 %, while revival of $S_{37°C}$ and $S_{20°C}$ populations was lower ($P \leq 0.05$) than 10 % at 14 °C. Germination of $S_{43°C}$ spores also displayed a lower pH boundary than the other two populations. These spores germinated up to ca. 10 % at pH 3.8, while $S_{20°C}$ and $S_{37°C}$ reached a similar level of germination between pH 4.5 and 4.2. We assayed a_w boundaries for germination in the range of 0.96 to 0.85 using NaCl, sucrose, or glycerol as a solute. As observed in Table 4, the threshold for the three populations was the highest in NaCl (a_w 0.96–0.94), followed by glycerol (a_w 0.96–0.88), and all spores were capable of germinating at a_w as low as 0.85 (saturation point) using sucrose. $S_{43°C}$ spores were again the ones with the lowest germination boundary in both NaCl and glycerol. Depressing a_w to 0.94 with NaCl and to 0.88 with glycerol virtually inhibited $S_{37°C}$ and $S_{20°C}$ germination, whereas $S_{43°C}$ spores still presented a germination efficiency of 23.7 % and 18.7 %, respectively.

Therefore, $S_{43°C}$ were not only the spores which presented the highest germination rate and/or efficiency in a great number of scenarios under optimal and adverse conditions, but they also displayed lower temperature and pH thresholds as well as a higher NaCl concentration threshold than their counterparts which had sporulated at optimal and suboptimal temperature. Boundaries for the inhibition of germination are generally lower than those for non-permissive growth (Jakobsen & Murrell, 1977; Rao et al., 2018; Smoot & Pierson, 1982); moreover, according to our results, that distance is larger in spores produced at supraoptimal temperatures. Further studies are needed to evaluate whether sporulation temperature also affects germination boundaries of food-borne pathogenic spores, thereby compromising the effectiveness of preservation barriers commonly implanted to ensure food safety.

Table 4
Temperature, pH, and a_w lower limits for germination of $S_{20°C}$, $S_{37°C}$, and $S_{43°C}$ spores in NBYE. Germination boundaries were set as conditions under which the percentage of germinated spores was ≤10 %.

Population	Temperature (°C)[a]	pH[b]	a_w (NaCl)[b]	a_w (sucrose)[b]	a_w (glycerol)[b]
$S_{37°C}$	≈14	4.0–4.2	0.96	<0.85*	≈0.88
$S_{20°C}$	≈14	≈4.5	0.96	<0.85*	≈0.88
$S_{43°C}$	<8*	≈3.8	<0.94*	<0.85*	<0.88*

[a] Samples were incubated for 7 days.
[b] Samples were incubated for 4 days.
* The percentage of germination lay above 10 %; however, more restrictive conditions could not be tested due to methodological difficulties.

4. Conclusions

This study demonstrated that sporulation temperature largely determines the germination dynamics of *B. subtilis* spores, and that such differences are exacerbated under certain food preservation stresses, even in nutrient-rich matrices. Thus, sporulation temperature, together with inherent strain-to-strain heterogeneity and, possibly, other sporulation conditions, should be regarded as an important source of variability when predicting spore responses. In our experimental setup, the most striking fact was that non-heat-activated spores produced at 43 °C had a better germination rate and/or efficiency than those obtained at 37 °C and 20 °C in a variety of nutrients under optimal environmental conditions, and that the advantageous germination fitness of $S_{43°C}$ spores was maintained under suboptimal temperature, pH, and high NaCl concentration in certain stimuli. Furthermore, in comparison with the other two populations, $S_{43°C}$ spores displayed lower temperature and pH boundaries and a higher NaCl concentration boundary for the inhibition of germination in a nutrient-rich medium. The assessment of spore responses in foods featuring populations produced under optimal sporulation conditions may therefore tend to underestimate the extent of germination if spores proceed from high-temperature environments. These outcomes encourage the pursuit of future studies regarding the influence of other sporulation conditions, such as pH or a_w, on germination dynamics. It would also be useful to determine whether our findings apply to other sporulated strains that play a relevant role in food safety and stability.

CRediT authorship contribution statement

Víctor Freire: Conceptualization, Formal analysis, Investigation, Methodology, Writing- original draft, Writing- review & editing. **Santiago Condón:** Conceptualization, Formal analysis, Supervision, Writing – review & editing. **Elisa Gayán:** Conceptualization, Formal analysis, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This study was supported by a grant [PID2019-104712RA-I00] funded by MCIN/AEI/10.13039/501100011033 and a PhD fellowship from the Government of Aragon (to V.F.).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114064.

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