Food Research International 221 (2025) 117248

Contents lists available at ScienceDirect

Food Research International

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

High salinity sporulation in *Bacillus subtilis* results in coat dependant enhanced resistance to both heat and hydrogen peroxide

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ARTICLE INFO

Keywords:
Sporulation water activity
Bacterial spores
Heat resistance
Salinity
Coat
Cross-link
Proteomics

ABSTRACT

In natural niches, spore-formers encounter non-optimal sporulation conditions that can affect spore resistance properties. However, while the influence of sporulation temperature on spore behaviour has been widely studied, knowledge concerning the influence of other factors such as water activity (a_w) is scarce. We previously reported that reducing the a_w of the sporulation medium from the optimum (a_w 0.99; $S_{control}$ spores) to 0.98 using sodium chloride (S_{salt} spores) increased the wet heat resistance of *Bacillus subtilis* 168 spores. The present work aimed to examine the mechanism behind the increased heat resistance observed in S_{salt} spores. The crust morphogenetic protein CotY was required for the increased heat resistance and increased resistance to H_2O_2 in S_{salt} spores. Label-free quantitative proteomics of $S_{control}$ and S_{salt} spores revealed markedly different protein profiles. Specifically, S_{salt} spores displayed significant increases in abundance of proteins involved in redox homeostasis, as well as changes in coat structural proteins, some of which are involved in protein–protein interactions. This work advances our understanding of how the coat modulates resistance in bacterial spores, helping develop effective control strategies against problematic spore populations due to occurring environmental changes.

1. Introduction

The extraordinary resistance of bacterial spores enables them to persist and disperse in the environment over long times and distances (Chen et al., 2010; Enger et al., 2018; Wijman et al., 2007). This makes them ubiquitous, rendering their entrance into the food chain almost unavoidable (Beskrovnaya et al., 2021). Once there, these spores withstand all but the most intense thermal sterilization treatments applied to foods. Survivors may then germinate in response to the nutrients present (André et al., 2017). Subsequently, germinated spores outgrow into vegetative cells, regaining the ability to multiply and produce toxins, ultimately compromising food stability and safety (André et al., 2017; Setlow & Johnson, 2019). While increasing the intensity of heat treatments may seem like a straightforward solution to mitigate these risks, thermal sterilization negatively impacts the organoleptic and nutritional qualities of foods (Sevenich & Mathys, 2018).

These facts have justified decades of research into the resistance mechanisms of bacterial spores against various agents, especially wet heat – the principal spore inactivation method used in the food industry - with the aim of developing alternative, cost-effective food processing methods that better preserve quality. The extraordinary resistance of spores is attributed to their unique structure and composition, consisting of superimposed layers that engulf a dehydrated core delimited by an inner membrane (IM). This dehydration is a fundamental factor explaining the several orders of magnitude difference in heat resistance between spores and their vegetative cell counterparts (Setlow, 2014). During sporulation, water content is partially replaced by dipicolinic acid chelated in a 1:1 ratio with Ca^{+2} (Ca-DPA), which compromises around 25 % of the spore's dry weight and contributes significantly to its resistance against wet heat (Kanaan et al., 2022; Setlow et al., 2006). Furthermore, the properties of the highly compact lipid bilayer of the IM have been shown to significantly contribute to resistance against heat (Berendsen, Boekhorst, et al., 2016; Berendsen, Koning, et al., 2016; Flores et al., 2023; Kanaan et al., 2022; Korza et al., 2023). Core dehydration is also maintained by the cortex, a modified peptidoglycan layer that compresses the IM. The cortex is surrounded by several layers of proteinaceous nature, the coat, which has the function to regulate entry of molecules, such as germinants, repel or neutralize chemicals, and resist phagocytosis (Ghosh et al., 2018; Kanaan et al., 2022; Klobutcher et al., 2006; Malyshev et al., 2021; Saggese et al., 2022). In *B. subtilis*, the coat is composed of several layers, arranged from outermost to innermost: the crust, the outer coat, inner coat, and basement layer

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https://doi.org/10.1016/j.foodres.2025.117248
Received 13 June 2025; Received in revised form 3 August 2025; Accepted 4 August 2025
Available online 7 August 2025

(McKenney et al., 2013). Importantly, the presence of this structure and the degree of cross-linking between coat proteins have been evidenced to influence heat resistance, and were hypothesized to be a consequence of IM stabilization during heat stress (Abhyankar et al., 2015; Kanaan et al., 2022).

Spore features related to heat resistance, such as core water content, IM properties, and coat composition, can be altered by the environmental conditions in which spores are formed (Beaman & Gerhardt, 1986; Bressuire-Isoard et al., 2016; Cortezzo & Setlow, 2005; Isticato et al., 2020). While the effect of sporulation temperature on spore heat resistance has been studied in detail (Bressuire-Isoard et al., 2018; Huang Roseboom et al., 2023; Melly et al., 2002), other highly influential factors such as water activity (a_w) have received less attention. This scenario may become increasingly plausible in the context of global warming and its associated effects on soil salinization (European Environment Agency (EEA), 2019; Intergovernmental Panel on Climate Change (IPCC), 2022) which incentives the study of spores formed under such conditions. We previously reported that *B. subtilis* 168 spores produced at a_w 0.98, adjusted using either glycerol or NaCl, were more resistant than those sporulated at optimal conditions (a_w 0.99; Freire et al., 2023). Notably, spores prepared under high salinity conditions showed increased resistance across a wider range of treatment temperatures and a reduced extent of sublethal injury (Freire et al., 2023). Furthermore, spores produced under high salinity conditions showed an heterogeneous germination response, (Freire et al., 2025). Therefore, spores contaminating foods that were formed under high salinity conditions may compromise food safety and stability, especially when processing parameters are based on challenge tests using spores produced under ideal laboratory conditions.

The persistent need to design heat-based treatments capable of effectively inactivating contaminating spores, regardless of their origin, requires a deep understanding of the cellular and molecular impact of environmental sporulation factors that drive increased spore resistance. In this context, the present research aims to elucidate the mechanisms underlying the enhanced heat resistance in *B. subtilis* spores, as well as their increased H_2O_2 resistance observed in this study, when sporulated at high salinity conditions.

2. Material and methods

2.1. Obtaining and purifying of spore suspensions

The wild type *B. subtilis* 168 strain (WT), was kindly provided by Prof. R. Kolter (Harvard University). Its deletion mutants Δ*cotY::erm* (Erm^R) and Δ*cotE::erm*(Erm^R), lacking CotY or CotE coat morphogenetic proteins, referred to in this work as Δ*cotY* and Δ*cotE*, respectively, were also used throughout this investigation. These deletion strains were constructed by SPP1 phage transduction as previously described (Burton et al., 2019), using as donor strains the deletion mutants (BKE11750 and BKE17030) from the BKE genome-scale deletion library (NBRP - National BIO-Resource Project, Japan) developed by Koo et al. (2017).

The strains were 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 (NAYE; Oxoid) and incubated at 37 °C for 24 h. Subsequently, 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; Heidolph Promax 1020, Schwabach, Germany). 20 μL of the culture were then transferred into a 250-mL flask containing 20 mL of liquid 2 × SG medium to obtain spores formed under optimal conditions ($S_{control}$) or 2 × SG medium with 2.75 % NaCl (Panreac, Barcelona, Spain) to obtain spores under high salinity conditions (S_{salt}; Freire et al., 2023). 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 previously described (Freire et al., 2023).

2.2. Quantification of DPA levels and wet density

Spore DPA content was determined by terbium fluorometry using autoclaved samples (121 °C, 20 min) to which $TbCl_3$ (Sigma-Aldrich, St. Louis, MI, USA) was added to a final concentration of 50 μM. Fluorescence was measured with a multiwell plate reader (CLARIOstar Plus, BMG, Ortenberg, Germany), using an excitation wavelength of 270 nm and an emission wavelength of 545 nm (Yi & Setlow, 2010). The background fluorescence was subtracted from all the samples, and a calibration curve of DPA (Sigma-Aldrich) was used to calculate DPA concentrations. These DPA concentrations were compared to the microscopic counts (spores/ mL) obtained using a Thoma counting chamber to obtain the mean DPA content/ per spore.

Wet density of spore populations was obtained by equilibrium density gradient centrifugation. In brief, 200 ul at a concentration of ca. 2×10^9 spores/mL were carefully layered on top of a Nycodenz® density gradient. The gradient was created as described in (Coleman, and D., Li, Y., Cowan, A. E., and Setlow, P., 2007). using PBS (Sigma-Aldrich) as solvent. Centrifugation to reach equilibrium was performed in a swinging bucket rotor at 3345 g for 60 min at 4 °C without brakes. This resulted in a well-defined band for each spore sample, aliquots from the region immediately superior to the equilibrium band, and immediately inferior region were taken. Subsequently, both the upper and lower aliquots were decimally diluted four times to calculate the spore density. This was done by comparing the OD_{244} arithmetic mean of the diluted samples from both regions, to the calibration curve made of Nycodenz solutions of known density.

2.3. Thermal treatment

To examine heat inactivation kinetics, treatments were carried out in a specially designed thermoresistometer at 105 °C in McIlvaine citrate-phosphate buffer of pH 7.0 (Dawson et al., 1986), as previously described (Condón et al., 1993; Freire et al., 2023). DPA release kinetics were assessed by treating samples at 95 °C in a PCR thermocycler (Bio-Rad T100, Hercules, CA, USA). Released DPA content was determined by terbium fluorometry, using autoclaved samples as the 100 % fluorescence reference, as detailed in (Freire et al., 2024).

2.4. Determination of survival

Survival was routinely determined by pour-plating in NAYE. NAYE plates were incubated at 37 °C for 24 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.

2.5. Calculation of heat resistance parameters

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.

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

2.6. Resistance to H_2O_2

Resistance to H_2O_2 was assessed as previously described (Kanaan et al., 2022; Korza et al., 2023), with minor modifications. Briefly, 10^8 spores / mL were incubated in 10 % H_2O_2 (Panreac) at 25 °C for inactivation, then diluted 1:10 into a catalase solution (Sigma-Aldrich) to neutralize remaining H_2O_2 prior to plating (Kanaan et al., 2022).

2.7. Label-free quantification (LFQ) proteomics

This process was realized by boiling the spores in a mixture of reducing agents SDS-DTT with the aim to solubilize the coat proteins as described previously by other authors in more detail (Isticato et al., 2020). The protein content of the extracts was measured using the RC DC Protein Assay Kit (Bio-Rad). Equal amounts of protein were loaded into each lane of precast 4–15 % polyacrylamide (Bio-Rad) gels prior to electrophoresis for initial assessment of differences.

For label-free quantification (LFQ) proteomics, samples were cleaned in-gel by SDS-PAGE, allowing them to run only until the proteins entered the top of the resolving gel. Subsequently, a single band was excised for each sample. The digestion of excised samples was carried out in an automatic digester (Intavis, Bioanalytical Instruments, Cologne, Germany). Briefly, the bands were washed with water, ammonium bicarbonate (100 mM), and acetonitrile (ACN). The samples were then reduced by incubation with DTT (10 mM) at 60 °C for 45 min and alkylated by incubation with iodoacetamide (50 mM) at room temperature for 30 min in the dark. Finally, proteins were digested with trypsin overnight at 37 °C (5 ng/µl, Trypsin Gold, Promega, Madison, WI, USA). The digestion was stopped by adding 0.5 % trifluoroacetic acid and the tryptic peptides were extracted sequentially with increasing concentrations of ACN in water.

Proteins were identified and quantified using a hybrid trapped ion-mobility quadrupole time-of-flight mass spectrometer (TIMS TOF Flex, Bruker Daltonics, Bremen, Germany) coupled online to an EvoSep ONE liquid chromatograph (EvoSep, Odense C, Denmark). Peptide quantification was carried out using the Qubit kit according to the manufacturer's instructions. A total of 200 ng of digested samples were directly loaded onto the EvoSep ONE chromatograph, and profiles were acquired using the 60 SPD (samples per day) protocol.

Peptides were separated on a C18 column (8 cm × 150 µm, 1.5 µm, Evosep) using a linear 21-min gradient and a cycle time of 24 min at a constant flow rate of 1 µL/min. Column temperature was controlled at 40 °C. Data were acquired using data-dependent acquisition mode with PASEF (parallel accumulation serial fragmentation). MS data were collected over an m/z range of 100 to 1700 and a mobility range of 0.60–1.60 V·s/cm^2. During each MS/MS data collection, each TIMS cycle lasted 1.1 s and included one MS and ten PASEF MS/MS scans.

Protein identifications and quantifications were performed using Bruker ProteoScape™ software and searched against the UniProt *Bacillus subtilis* protein database plus sequences of known contaminants (UP000001570). A precursor tolerance of 20 ppm and a fragment ion tolerance of 30 ppm were used. The search space included all fully- and half-tryptic peptide candidates with up to two missed cleavages. Carbamidomethylation (+57.02146) of cysteine was considered a static modification. TIMScore was enabled to incorporate peptide Collisional Cross Section (CCS) during the scoring process. Identifications were rescored using an updated version of MS2Rescore. These results were validated, assembled, and filtered using a false discovery rate (FDR) of 0.01; under these filtering conditions, the estimated FDR was below ~1 % at the protein level in all analyses. Data were filtered to remove proteins with fewer than two peptides, as well as contaminants and reverse hits. The cleaned, quantified protein data file was statistically analyzed and visualized using Perseus (version 2.0.3.0) (Tyanova et al., 2016). Five biological replicates obtained under different working days were included for the LFQ assays.

2.8. Statistical analysis

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

3. Results

3.1. Increased resistance of S_{salt} spores is not associated with changes in DPA content or wet density

The main established factor contributing to spore heat resistance is low core water content, partially replaced by dipicolinic acid (DPA), which stabilizes essential structures such as the core and membrane-associated metabolic machinery (Beaman et al., 1982; Kanaan et al., 2022; Setlow et al., 2006). Given that water and DPA content are known to change with environmental sporulation conditions (Beaman & Gerhardt, 1986; Bressuire-Isoard et al., 2018), suspensions obtained under both optimal ($S_{control}$) and high salinity (S_{salt}) sporulation conditions were examined for changes in DPA content and wet density. In this regard, no significant differences ($P > 0.05$) were found in the amount of DPA content per spore, with around 0.8 ± 0.08 picograms of DPA per spore, regardless of sporulation condition. Similarly, no significant differences arose among sporulation conditions for wet density, with density values of around 1.3 g/cm^3 (Fig. 1). The DPA content and the wet density observed is in accordance to data reported for *B. subtilis* spores obtained under optimal sporulation conditions in the literature (Jamroskovic et al., 2016; Rose et al., 2007). These results indicated that the increase in wet heat resistance exhibited by S_{salt} spores was unlikely to be due to lower water content or increased Ca-DPA mediated stabilization during heat stress.

3.2. Changes in the coat induced by sporulation at high salinity conditions are associated with increased heat resistance in S_{salt} spores

It has been shown that changes in coat composition, modulated by environmental sporulation conditions, influence spore heat resistance to heat (Bressuire-Isoard et al., 2018; Isticato et al., 2020). In this sense, sporulation of *Bacillus subtilis* 168 at high salinity was found to influence nutrient germination and coat permeability (Freire et al., 2025). Given the evidence of coat structural modifications under high salinity conditions, we evaluated whether this structure also contributed to the increase in wet heat resistance.

Survival curves at 105 °C of spores from the WT strain, Δ*cotY* mutant (in which the crust is disrupted; Bartels et al., 2019; Freire et al., 2025), and Δ*cotE* mutant (lacking both the crust and the outer coat; Chada et al., 2003; McKenney et al., 2013), sporulated under both optimal and high salinity conditions, are shown in Fig. 2. For comparison, inactivation kinetics were modeled, and the resulting resistance parameters are detailed in Table 1. As expected (Freire et al., 2023), S_{salt} WT spores displayed a higher wet heat resistance, characterized by an increased shoulder length, lower inactivation rate (k_{max}) and an overall higher $3D_{105\ °C}$ value in comparison to $S_{control}$ WT spores (Table 1, $P \leq 0.05$,). Conversely, inactivation parameters of $S_{control}$ Δ*cotY* and S_{salt} Δ*cotY* spores did not show statistically significant differences ($P > 0.05$; Table 1) between each other or when compared to $S_{control}$ WT spores. On the other hand, the $3D_{105\ °C}$ values of Δ*cotE* spores were significantly inferior to those values observed for WT or Δ*cotY* spores, regardless of sporulation condition (Fig. 2, Table 1). Inactivation kinetics of $S_{control}$ Δ*cotE* and S_{salt} Δ*cotE* spores were identical ($P > 0.05$; Fig. 2, Table 1). Interestingly, even though a higher inactivation rate was appreciated, these differences were mainly caused by the reduction in the shoulder length which was around 4-fold lower in Δ*cotE* spores than that of

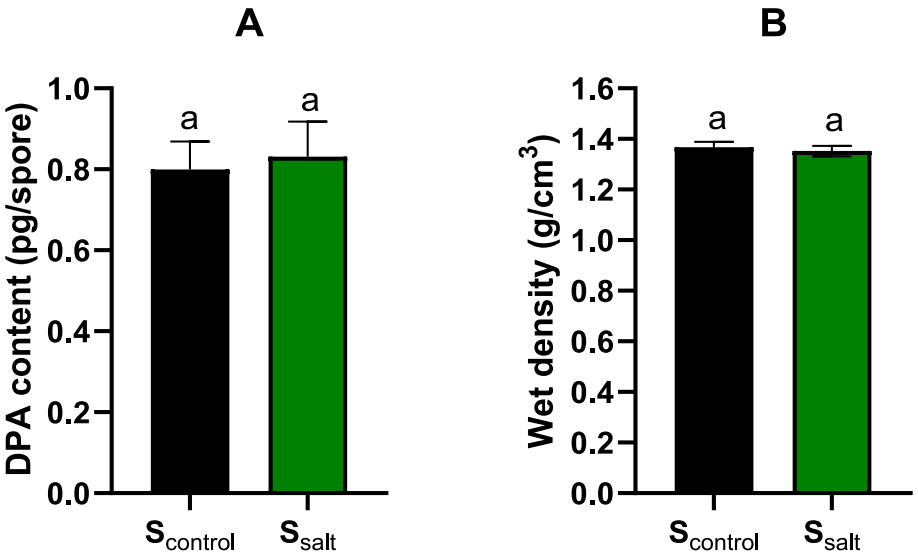

Fig. 1. DPA content per spore (A) and wet density (B) of *B. subtilis* spores obtained under optimal ($S_{control}$, black bars) and high salinity conditions (S_{salt}, green bars). Different letters indicate statistically significant differences ($P \leq 0.05$) among sporulation conditions for each property assayed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

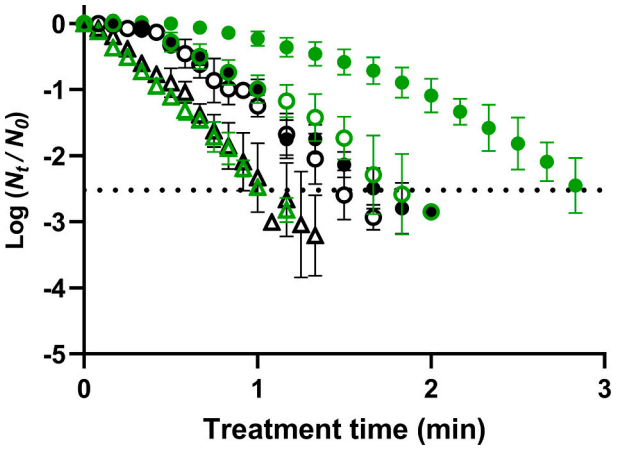

Fig. 2. Survival curves at 105.0 °C of spore *B. subtilis* spores produced under optimal (black symbols) and high salinity conditions (green symbols). WT spores are depicted with full symbols (●), whilst coat defective strains are depicted by empty symbols; Δ*cotY* (○) and Δ*cotE* (Δ), respectively. The dotted line represents the limit of quantification (30 CFU/ plate).

Table 1
Heat resistance parameters of the different spore populations obtained from the fit of the Log-lineal + shoulder model (Geeraerd et al., 2000) to survival curves at 105 °C (Fig. 2). Data in brackets represent the standard deviations of the means. Letters indicate statistically significant differences ($P \leq 0.05$) among strains sporulated at the same condition (optimal or increased salinity medium), while an asterisk indicates statistically significant differences ($P \leq 0.05$) between the two sporulation conditions within each strain.

Spore population	*Sl* (min)	k_{max} (min^{-1})	$3D_{105}$ (min)	R^2	RMSE
WT $S_{control}$	0.42^{a} (0.04)	4.42^{a} (0.46)	1.99^{a} (0.08)	0.983	0.171
Δ*cotY* $S_{control}$	0.50^{a} (0.06)	5.35^{a} (0.38)	1.59^{ab} (0.22)	0.993	0.065
Δ*cotE* $S_{control}$	0.17^{b} (0.08)	7.33^{c} (0.61)	1.12^{b} (0.21)	0.996	0.063
WT S_{salt}	1.22^{c*} (0.18)	3.30^{b*} (0.31)	3.32^{c*} (0.29)	0.993	0.066
Δ*cotY* S_{salt}	0.57^{a} (0.19)	4.77^{a} (0.73)	2.04^{a} (0.19)	0.977	0.135
Δ*cotE* S_{salt}	0.07^{b} (0.04)	5.94^{c} (0.29)	1.24^{b} (0.07)	0.992	0.072

$S_{control}$ WT spores ($P \leq 0.05$, Table 1).

The consensus from previous studies is that DPA release is correlated with heat resistance and structural integrity, most likely of the IM, although this correlation is influenced by strain and treatment conditions (Coleman Chen et al., 2007; Kort et al., 2005; Setlow et al., 2006; Setlow & Christie, 2023; Tu et al., 2021). To further validate the involvement of CotY in the enhanced heat resistance, we assessed the DPA release from WT and Δ*cotY* spores produced under optimal and high salinity conditions during heating at 95 °C (Fig. 3). Once more, increased wet heat sensitivity was observed in $S_{control}$ spores when compared to S_{salt} WT spores while deletion of CotY eliminated these differences among sporulation conditions (data not shown). Consistent with their increased survival, S_{salt} WT spores showed a slower DPA release than $S_{control}$ WT spores. After 45 min S_{salt} WT spores released 40 % of its total DPA content, significantly lower than the 59 % released by $S_{control}$ WT spores during the same treatment ($P \leq 0.05$; Fig. 3). In addition, Δ*cotY* spores, regardless of sporulation condition, liberated around 53 % during the same treatment time, showing no statistical

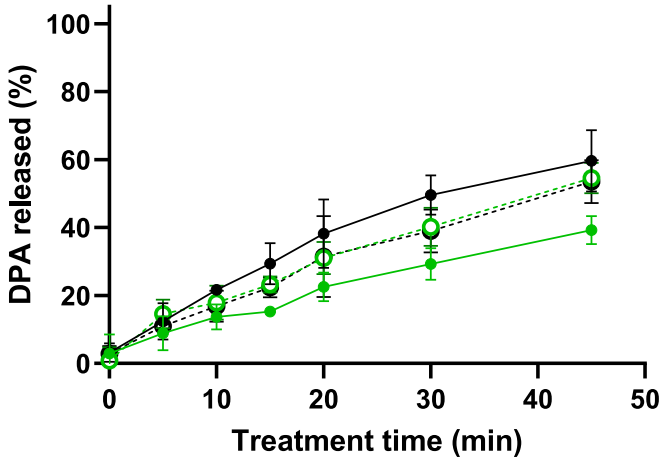

Fig. 3. Kinetics of DPA release at 95 °C from WT and Δ*cotY* spores produced under optimal (black symbols) and high salinity conditions (green symbols). WT spores are depicted with full symbols and continuous lines (●), whilst Δ*cotY* spores release are depicted with empty symbols (○) and discontinued lines, respectively.

differences with the content released by $S_{control}$ WT spores ($P > 0.05$; Fig. 3). Therefore, these results confirmed that CotY play a critical role in the development of heat resistance in spores produced under high salinity conditions.

3.3. Changes in the crust induced by sporulation at high salinity conditions are associated with increased H_2O_2 resistance in S_{salt} spores

It has been suggested that both wet heat and H_2O_2 could kill spores of *B. subtilis* by similar mechanisms, inactivating a key target in the IM (Korza et al., 2023). In addition, hydrogen peroxide is a disinfection agent widely used in the food industry due to its broad-spectrum antimicrobial activity against bacteria, fungi, viruses, and spores, and finally, ability to breaking down into water and oxygen, leaving no toxic residue (Abdelshafy et al., 2024). Therefore, due to its mechanistical and on the other hand, practical relevance in food environments, the resistance to this chemical of the WT and Δ*cotY* spores produced under optimal and high salinity conditions was examined.

H_2O_2 treatment for 20 min led to inactivation of ca. 2.3 log cycles of $S_{control}$ WT population, conversely, mean inactivation induced for S_{salt} WT population was significantly less, of ca. 0.3 log cycles ($P \leq 0.05$, Fig. 4). On the other hand, $S_{control}$ Δ*cotY* spores were inactivated around 1.8 cycles while S_{salt} Δ*cotY* spores suffered a decrease in the viability of the population of around 1.4 cycles, hence showing no significant differences among them ($P > 0.05$, Fig. 4). Precisely, the fact that inactivation of $S_{control}$ Δ*cotY* suspensions was not statistically different from that of $S_{control}$ WT spores, while, conversely, spores obtained at high salinity were statistically more sensitive to H_2O_2 in spores lacking a complete crust (S_{salt} Δ*cotY*) suggests that a significant modification of the coat, dependent of CotY in S_{salt} spores granted these spores a considerable resistance increase to H_2O_2 as well. However, as S_{salt} Δ*cotY* still maintained increased resistance over WT $S_{control}$ spores, these results suggest that additional modifications, may also participate in the increased resistance to H_2O_2 (see below and Discussion). In any case, these results indicated that use of this disinfection agent against spores formed under high salinity may result in 100- fold increased risk of survival compared to spores formed under optimal conditions.

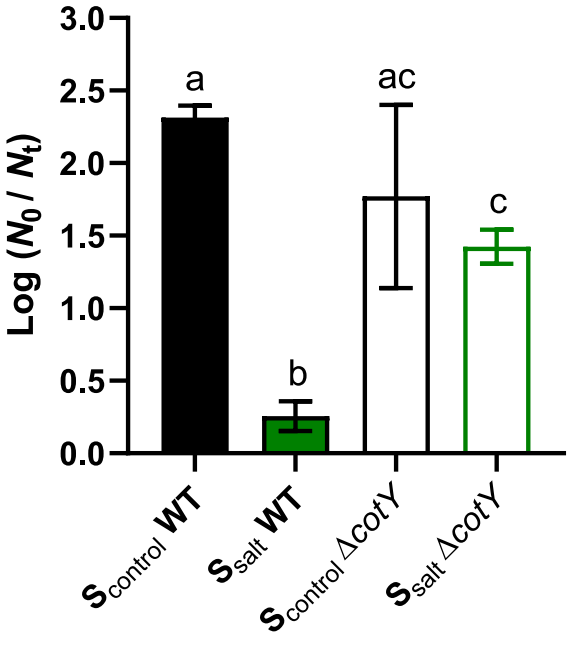

Fig. 4. Resistance of $S_{control}$ (Black) and S_{salt} (green) spores (Full bars, WT and empty bars Δ*cotY*) against 10 % H_2O_2 for 20 min. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Spores obtained under high salinity conditions suffer significant changes in coat protein composition

Consequently, we hypothesized that changes in the coat that required CotY were behind the increased resistance of S_{salt} spores. In order to gather further information, we decided to assay protein extraction of the coat by SDS-DTT procedure as previously described (Isticato et al., 2020). Importantly, SDS-PAGE gel anticipated differences in the solubilized coat proteins between $S_{control}$ and S_{salt} WT spores (Fig. S2). More specifically, the intensity of three bands of sizes around 65 kDa, 50 kDa, and 30 kDa visibly in $S_{control}$ spores diminished in S_{salt} spores. However, the complex nature of the protein-protein interactions within the coat can cause some proteins to be co-extracted with their interacting partners, making identification by this method unreliable (Isticato et al., 2008). Therefore, we opted to subject the coat extracts to label free identification and quantification proteomic analysis to better determine the changes suffered by the coat during sporulation under high salinity conditions.

The LFQ proteomic data, depicted in Fig. 5, showed major alterations in the coat composition between S_{salt} and $S_{control}$ spores. Interestingly, one of the most prominent changes provoked by sporulation at high salinity was significant increase in the abundance of proteins involved in redox homeostasis, particularly the thiol-peroxidase (Tpx), the disulfide bond formation protein (DbdB), the superoxide dismutase (SodA), and the thioredoxin (TrxA) (Fig. 5, blue dots). This suggest that sporulating cells experience higher oxidative stress when exposed to elevated salinity in the sporulation medium compared to optimal conditions. This observation concurs with results obtained by other studies conducted examining *Bacillus* species, observing induction of peroxidases and superoxide dismutases under salinity stress (Goosens et al., 2013; Hassan et al., 2020). In addition, YneT was also found to be significantly increased in S_{salt} populations. Remarkably, this protein is known to undergo CoAlation in a CotE- and GerE-dependent manner, although its function remains unclear (Zhyvoloup et al., 2020).

In addition, coat structural proteins showed increased abundance in S_{salt} spores (Fig. 5, green dots), including proteins within every layer: basement (SpoVID, YheD), inner coat (SafA), outer coat (CotB, CotM, CotD), and crust (CotW, CotX, and CgeA). Conversely, coat kinase proteins such as CotSA and CotS were upregulated in $S_{control}$ spores (Fig. 5, black dots). Therefore, a major change in structural coat components occurs due to sporulation at high salinity. However, no significant increase was observed in the abundance of the crust morphogenetic proteins CotY. Since this protein was necessary for the development of the increased resistance phenotypes caused by sporulation at high salinity (Fig. 2 and Fig. 3), this could point to protein-protein interaction as the cause for this behaviour.

As a consequence, we examined which of these proteins found in the samples were known to interact with themselves or other coat constituents. Sixteen proteins significantly more abundant in S_{salt} spores and one protein more abundant in $S_{control}$ spores (CotQ) have been demonstrated to participate in protein-protein interactions (Table 2). For instance, proteins such as BdbD (Bacillus disulfide bond D) and SodA, are known to promote oxidation of thiol groups, such as those present in cysteine residues, contributing to the formation of disulfide bonds in secreted proteins and, in the case of the former enzyme, cross-link of coat proteins, aiding to their correct folding and stability (Crow et al., 2009; Henriques et al., 1998). In this sense, two proteins experimentally demonstrated to stablish disulfide bonds in the coat, CotJA and CotJC (Ursem et al., 2021), presented higher abundance in S_{salt} spores. Furthermore, sporulation under high salinity conditions resulted in increased levels of proteins involved in dityrosine cross-links, such as CotJB and SpsB (Ursem et al., 2021). Finally, some enzymes involved in maturation of the coat structure and acquisition of definitive germination properties through the formation of ε-(γ-glutamyl) lysine bonds, such as TgL and YabG (Fernandes et al., 2018; Fernandes et al., 2019; Monroe & Setlow, 2006; Ragkousi & Setlow, 2004), were also enriched

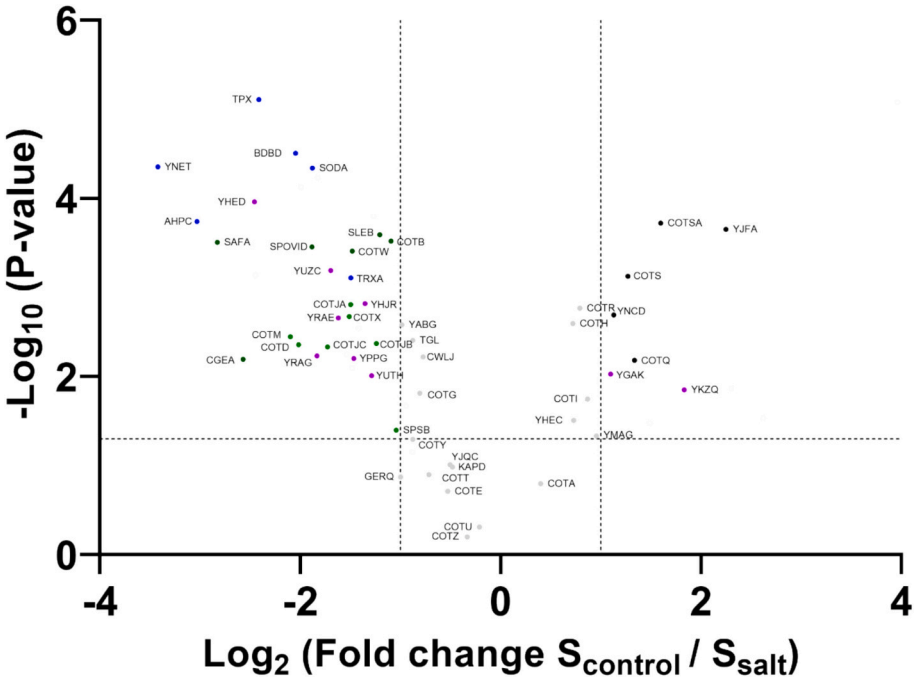

Fig. 5. Volcano plot of differentially abundant coat structural or resistance related proteins between S_{salt} and $S_{control}$ spores. Horizontal line delimits the $P = 0.05$ statistically significant level. Vertical lines indicate a Log_2 Fold Change (FC) in abundance of proteins between both populations. Grey proteins outside these regions are below the FC and *P*-value cut-off to be considered significant. Blue proteins are proteins involved in redox homeostasis and disulfide bond formation. Dark green dots are known structural coat proteins of statistical higher abundance in S_{salt} spores. Black dots are known structural coat proteins of statistical higher abundance in $S_{control}$ spores. Purple proteins are coat proteins of unknown function of statistical higher abundance in either population. Depicted data were calculated from five biological replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in S_{salt} populations, although their fold changes were slightly inferior to 2-fold.

In conclusion, these results show that the upregulation of proteins involved in redox balance, along with changes in coat structural proteins and the nature and extent of protein-protein interactions, including those known to directly interact with CotY may contribute to coat modifications that enhance heat and H_2O_2 resistance in S_{salt} spores.

4. Discussion

The effect of sporulation conditions, and specially sporulation temperature, is widely recognized as an important factor contributing to variability in spore resistance (Bressuire-Isoard et al., 2018; Freire et al., 2023). However, the impact of a_w has been overlooked so far, despite the fact that environments with lower humidity than ideal culture media, such as soil, are common sporulation niches (Carlin et al., 2010). This issue is especially relevant given the increasing salinization of soils as a consequence of global warming (European Environment Agency (EEA), 2019; Khamidov et al., 2022). Available reports indicate that in the Mediterranean region, approximately 25 % of cropland is affected by salinization (Geeson et al., 2003; Mateo-Sagasta & Burke, 2011). For example, in Spain 3 % of the 3.5 million hectares of irrigated agricultural land, is severely affected, and an additional 15 % is at serious risk (Stolte al., 2016). Our previous research demonstrated that *B. subtilis* populations sporulated at depressed a_w (0.98) by the addition of NaCl (S_{salt}) exhibited increased heat resistance compared to spores incubated under optimal conditions (a_w 0.99, $S_{control}$). The fact that thermal processing is the predominant food preservation technology, along with the foreseeable increase in prevalence of spores formed under high salinity natural environments, prompted us to investigate the mechanisms underlying the enhanced resistance of S_{salt} spores. Indeed, understanding the mechanisms by which environmental sporulation conditions contribute to the development of spore heat resistance is essential for designing more effective eradication strategies in the food industry. $S_{control}$ and S_{salt} spores posed similar DPA content and wet density, both features previously associated with increased heat resistance observed in spores formed at higher temperatures Beaman et al., 1982; Beaman & Gerhardt, 1986; Marquis & Bender, 1985; Palop et al., 1999). Thus, it was unlikely that sporulation at high salinity increased wet heat resistance through either of these mechanisms.

Alternatively, increased wet heat resistance could still be explained by modifications in other structural components, such as the coat and the IM, which are also known to play a significant role, probably in conjunction (Kanaan et al., 2022; Korza et al., 2023). As coat alteration were implicated in the impaired nutrient germination of S_{salt} spores (Freire et al., 2025), we focused on the role of the coat in the development of heat resistance in spores produced under high salinity conditions. The inactivation kinetics of Δ*cotY* spores produced under both sporulation conditions were equal and indistinguishable from those observed for $S_{control}$ WT. In addition, loss of additional coat material occurring in Δ*cotE* spores further decreased spore heat resistance, yet again without showing any difference between sporulation conditions. This further decrease in heat resistance was caused by disappearance of the shoulder phase related with accumulation of sublethal damage (Condón et al., 1996; Feeherry et al., 1987). These results are supported by previous findings highlighting the importance of the coat in spore heat resistance either through coat-deficient mutants or chemical decoating treatments (Ghosh et al., 2008; Kanaan et al., 2022). Data from several authors, as well as our own, suggest that the coat stabilizes the membrane during heat stress by exerting pressure over the cortex and innermost layers which is critical for maintaining low permeability and for shielding embedded proteins that are crucial for heat survival (Kanaan et al., 2022; Setlow & Christie, 2023). Nevertheless, during lethal heat stress at 95 °C, final DPA release, as a consequence of loss of IM integrity (Coleman Chen et al., 2007), was greater in $S_{control}$ WT spores than in S_{salt} WT spores, while in Δ*cotY* spores release kinetics were identical among sporulation conditions, and at the same time undistinguishable to $S_{control}$ WT release ($P > 0.05$). It is important to

Table 2
List of coat and coat-related proteins involved in protein-protein interactions. Interaction types: S—S (disulfide), CoA (CoAlation), Y—Y (dityrosine), Q-K (ε-(γ)-glutamyl-lysine), and P—P (undetermined). Statistical significance: $P < 0.05$ (*), 0.01 (**), 0.001 (***), or 0.0001 (****). Data were calculated from five biological replicates. MGP: Morphogenetic protein. ATP—B: ATP-binding. Underlined proteins have been experimentally shown to interact directly with CotY.

Name (Uniprot)	Log_2 FC: $S_{control}/S_{salt}$	Interaction	Comment	References
YneT (Q45065)	−3.42 ****	CoA		(Zhyvoloup et al., 2020)
BdbD (O32218)	−2.05 ****	S-S	Thiol-disulfide oxidoreductase, required for the formation of thiol disulfide bonds in several proteins.	(Henriques et al., 2004; Henriques & Moran, 2007)
SodA (P54375)	−1.88 ****	P-P	Relevant for coat assembly, H_2O_2 production.	(Henriques et al., 1998)
SafA (O32062)	−2.83 ***	P-P	Self-interacts, coat MGP.	(Costa, et al., 2006; Kim et al., 2006)
YheD (O07545)	−2.46 ***	P-P	Self-interacts, ATP-B	(Krajčíková et al., 2021)
SpoVID (P37963)	−1.88 ***	P-P	Coat MGP, ATP-B	(Costa et al., 2006; Kim et al., 2006)
CotW (Q08310)	−1.48 ***	P-P	Anchoring of the crust to the outer coat.	(Bartels et al., 2019; Krajčíková et al., 2017)
CotB (P07789)	−1.09 ***	P-P	Self-interacts, phosphorylated by CotH.	(Abhyankar et al., 2015; Krajčíková et al., 2017)
CotJA (Q45536)	−1.50 **	S-S		(Ursem et al., 2021)
CotJB (Q45537)	−1.24 **	Y-Y		(Ursem et al., 2021)
CotJC (Q45538)	−1.73 **	S-S	Putative coat catalase.	(Ursem et al., 2021)
CotX (Q08313)	−1.51 **	S-S	Coat MGP.	(Krajčíková et al., 2017; Ursem et al., 2021; Zhang et al., 1993)
CotM (Q45058)	−2.10 **	P-P	Self-interacts.	(Krajčíková et al., 2017)
CotD (P07791)	−2.02 **	P-P		(Krajčíková et al., 2017)
CgeA (P42089)	−2.57 **	P-P	Self-interacts, glycoprotein.	(Krajčíková et al., 2017)
SpsB (P39622)	−1.04 *	Y-Y / Q-K	Putative glycosyltransferase.	(Ursem et al., 2021)
CotQ (O06997)	1.34 **	P-P		(Krajčíková et al., 2017)

note that DPA content of both Δ*cotY* and Δ*cotE* spores, showed no substantial differences when compared to WT spores, regardless of sporulation condition (Fig. S1). As a result, these findings suggested that CotY-dependent stabilization of the critical target preventing spore death and DPA release during lethal heat treatments occurs as a consequence of sporulation under high salinity conditions. This finding aligns with our previous observations that S_{salt} spores incurred a lower extent of sublethal damage than $S_{control}$ spores, as evidenced by selective media plating (Freire et al., 2023). Finally, TEM observations of *cotXYZ* deletion mutant spores have shown that the crust contributes to the tight packaging of the underlying layers (Freitas et al., 2020; Zhang et al., 1993).

Hydrogen peroxide is a disinfection agent widely used in the food industry due to its broad-spectrum antimicrobial activity, including spores (Abdelshafy et al., 2024). In this context, the killing efficacy of 10 % H_2O_2 for 20 min at room temperature against *S. salt* spores was negligible, resulting in only a 0.3-log reduction. This concentration lies at the upper end of those typically used in food industry applications such as whole-room disinfection, where concentrations between 1 % and 5 % are applied for 1 to 30 min (Abdelshafy et al., 2024; Møretrø et al., 2019). Despite this, the survival risk of S_{salt} populations increased 100-fold when compared to spores formed under optimal environmental conditions that may be used for biocide challenge tests. It has been suggested that the IM or its components are the critical targets for spore inactivation by H_2O_2, and that the coat offers some protection against its action (Kanaan et al., 2022; Korza et al., 2023; Leggett et al., 2016). In this regard, a Δ*cotE* deletion mutant of *B. subtilis* (PS533), lacking both the crust and the outer coat, showed decreased H_2O_2 resistance compared to parental spores against 1 % hydrogen peroxide (Leggett et al., 2016). Furthermore, chemical decoating also increased the sensitivity of spores to this agent, for example, 40 min of exposure to 11 % H_2O_2 led to a viability difference of over 3 log units between intact and chemically decoated spores of *Bacillus subtilis* PS533 (Kanaan et al., 2022). Therefore, it seems that regardless of using a high or a low concentration of this agent, the coat offers some protection against its action to preserve spore viability. In agreement, our results demonstrate that disruption of the crust by the absence of CotY already rendered spores more sensitive. Regarding the influence of sporulation salinity, S_{salt} WT spores showed increased resistance compared to $S_{control}$ WT spores, but this difference between sporulation conditions was negligible in the *cotY* deficient mutant, as also occurred with heat resistance. The enhanced protection conferred by sporulation under high salinity may be explained by reduced penetration of H_2O_2 into the IM, possibly due to an increased detoxifying capacity of specific coat components, such as redox enzymes (Hullo et al., 2001). In this way the coat may function as a "reactive armor", neutralizing the biocide before it can penetrate further into the spore at the cost of integrity (Driks, 1999).

Finally, it should be noted that the coat has also been shown to protect against other disinfection agents, such as sodium hypochlorite and UV-C radiation (Kanaan et al., 2022; Korza et al., 2023; Clair et al., 2020). However, S_{salt} spores were equal or more sensitive to these agents (Freire et al., 2023), indicating that sporulation under high salinity may significantly alter the efficacy of decontamination procedures depending on the agent or technology used.

The question of how actually CotY in *B. subtilis* could increase resistance against both wet heat and H_2O_2 led us to study the composition of this structure in both spore populations. Indeed, in-depth proteomic analysis revealed that shifting sporulation conditions from optimal to high salinity provoked significant changes in the abundance of coat proteins involved in redox homeostasis and their structural components. Furthermore, some other enzymatic components of the spore coat, such as kinases, which may influence structural properties and consequently germination (Saggese et al., 2022) also showed altered abundancy depending on the sporulation conditions.

Remarkably, CotY, which was needed for the increased heat and H_2O_2 resistance of S_{salt} WT spores, showed no significant differences in its abundance. These findings point to interactions among spore coat components, rather than simply an increased amount of CotY in the coat of spores produced under high salinity conditions, as the responsible for the increased resistance observed. Among plausible protein interactions, a varying degree of cross-linking between coat proteins may account for the differences in heat and H_2O_2 resistance induced by the two sporulation conditions studied. In *B. subtilis*, the crust layer is known to sustain extensive cross-linking—likely through disulfide bonds formed between cysteine-rich proteins encoded by the *cotVWXYZ* cluster (Ursem et al., 2021; Zhang et al., 1993)—with CotY being the component involved in the highest number of interactions with other coat proteins, including self-interactions (Krajčíková et al., 2017). Changes in the degree of cross-linking between coat proteins have been associated with variations in spore resistance. For example, increased cross-linking during spore

maturation has been reported to correlate with enhanced heat resistance (Abhyankar et al., 2015; Sanchez-Salas et al., 2011). In some instances, spores with significative differences in heat resistance due to varying sporulation from liquid to solid media have also been reported to show altered coat protein expression and cross-linking degree (Abhyankar et al., 2016). Furthermore, it was observed long time ago that pre-treatment with agents rupturing disulfide bonds rendered *B. cereus* spores more susceptible to H_2O_2 (Gould & Hitchins, 1963). In *Clostridium difficile*, cysteine-rich proteins of the outermost coat layers, such as CdeC, are required for spore heat resistance and also contribute to resistance against phagocytic cells, which exert antimicrobial activity through mechanisms including the production of reactive oxygen species like H_2O_2 (Calderón-Romero et al., 2018).

The proteomic differences in coat composition induced by sporulation under high salinity conditions suggest that increased cross-linking reactions in S_{salt} spores may contribute to their higher heat and H_2O_2 resistance compared to $S_{control}$ spores. First, some enzymes involved in the formation of all the three types of cross-links described in the *B. subtilis* spore coat—disulfide, dityrosine and ε-(γ-glutamyl) lysine bonds—were significantly more abundant in S_{salt} spores. For example, BdbD and SodA are either involved in or promote cross-linking reactions between coat proteins (Henriques et al., 1998; Krajčíková et al., 2017). Although the involvement of the former protein in the proper folding of secreted cysteine-containing proteins has been shown, it was not required for the development of resistance to mild heat treatment (80 °C for 15 min) in *B. subtilis* spores (Erlendsson et al., 2004). Whether it can provide protection against more intense treatments remains unknown. The absence of SodA has shown to cause major coat structural alteration (Henriques et al., 1998). In fact, the H_2O_2 produced by this superoxide dismutase can react with amino acids involved in coat cross-linking such as cysteine and lysine (Finnegan et al., 2010; Ursem et al., 2021). Lastly, SodA has been found to cross-link proteins such as CotG, although no significative effect on heat resistance of spores of strain AH1490 with no measurable superoxide dismutase activity was found (Henriques et al., 1998). In this context, it is important to note that the production of H_2O_2 by this enzyme family, and therefore, its ability to crosslink proteins, depends on the presence of superoxide radicals, which are more abundant under oxidative conditions, such as high salinity (Goosens et al., 2013;Hassan et al., 2020; Höper et al., 2006). In any case, the contribution of BdbD and SodA, especially the latter and its role in H_2O_2 production, by affecting cross-linking of proteins rich in these amino-acids, such as CotY is a tempting explanation that remains open for further investigation. In addition to these two proteins, the coat transglutaminase TgL, known to favour cross-linking by ε-(γ-glutamyl) lysine bonds (Sanchez-Salas et al., 2011), was also more abundantly present in S_{salt} spores, although the differences did not meet the cut-off criteria of FC $>$ 2, potentially suggesting a more limited contribution to the observed phenotype.

Second, several proteins involved in various types of physical protein-protein interactions within the coat of *B. subtilis*, including some that interact directly with CotY or self-interact, were also more abundant in S_{salt} than in $S_{control}$ spores. For instance, increased presence of some of the proteins involved in polymerization control and scaffolding of the coat structure such as SpoVID and SafA may also contribute to a more robust coat structure (Costa et al., 2006; Ozin et al., 2000). In this regard, the cysteine protease YabG has been shown to interact with both SpoVID and SafA, supporting coat assembly and resistance (Takamatsu et al., 2000). However, the essential role of CotY observed in our experiments supports the importance of proteins that either directly interact with it or are located in its immediate vicinity. This could be the case for CotW, CotX, CotM, and/or CgeA (Krajčíková et al., 2017; Liu et al., 2016), all of which showed an increase in their FC higher than 1.5 in S_{salt} (Table 2, underlined proteins). In this context, the more abundant increased CotW may function as an anchor between the crust and the rest of the spore coat structure (Bartels et al., 2019). Similarly, CotX was proposed to contribute to the physical attachment of the crust to the rest of the coat by Shuster et al. (2019). These authors also observed strong interactions among CotX, CotY, and CotW: CotY localization depended on CotW and co-depends on CotX, while CotX itself is stabilized by CotW. Likewise, CgeA localization also relied on both CotX and CotY. Importantly, CotX physical interaction with CotY was confirmed by single-molecule force spectroscopy (Liu et al., 2016).

Finally, there is evidence that proteins located on the spore surface can undergo spontaneous disulfide bond formation under oxidative conditions (Richter et al., 2015). As previously mentioned, salinity favours oxidative stress (Hassan et al., 2020; Höper et al., 2006), which may help explain the vital role of a crust protein such as CotY in developing the increased resistance observed in S_{salt} spores. Furthermore, oxidative conditions could also account for the increased presence of redox homeostasis proteins in these spores, either as structural components of the coat or potentially being adsorbed within it, as has been observed with heterologous proteins in *B. subtilis* (Huang et al., 2010; Isticato et al., 2013). In fact, other proteomic studies have also revealed an important presence of several redox-related proteins, such as Tpx or SodA, located in the outer coat (Abhyankar et al. (2015), supporting a regulatory role in the assembly and/or function of this structure, as suggested by other authors (Henriques et al., 2004). Therefore, the increased abundance of redox proteins such as CotJC, Tpx, TrxA, AhpC, SodA, and YneT in S_{salt} spores may help prevent the overoxidation of key coat proteins and/or protect critical structures such as the IM from oxidative damage in spores, although this is yet to be studied. Beyond their potential roles and regulatory networks, further studies are needed to determine the specific contribution of coat cross-linking and coat redox proteins to resistance against disinfection agents and heat.

Advancing our understanding in this area will improve our ability to decipher the resistance mechanisms of spores and support the development of novel control strategies in the food industry. For instance, compounds aiming at weakening the coat integrity or disrupt disulfide bonds may, based on our results, counteract the variability in heat resistance posed by sporulation conditions and reduce the intensity of heat treatments required for spore inactivation or enhance the effectiveness of biocides. In this context, the lethality of heat treatment against *Bacillus subtilis* spores was increased by an additional log reduction in viability when heating at 100 °C for 60 min was combined with 0.25 g/L of ε-polylysine, an FDA-approved additive. This combination induced greater structural disorder within the spores, including disruption of the spore coat, during heat exposure (Bi et al., 2023).

5. Conclusions

Due to the ongoing trend of soil salinization, spores formed under high salinity conditions may become increasingly prevalent in the food chain. In *B. subtilis*, sporulation under high salinity conditions resulted in spores with enhanced resistance to heat and to H_2O_2. The increased resistance to both agents was found to be dependent on the crust morphogenetic protein CotY. Salinity, likely linked not only to osmotic but also oxidative stress, resulted in the increased abundance of coat-associated redox homeostasis proteins such as Tpx and SodA, as well as several structural coat proteins involved in promoting or establishing cross-linking interactions, including those involving CotY. These modifications may confer structural resilience to the spore coat increasing survival risk to heat and H_2O_2 up to 100-fold. This highlights the importance of considering sporulation salinity when producing spore populations for challenge tests of heat or disinfection procedures. Furthermore, this study remarks the relevance of the coat as a source of variability in spore resistance to heat and disinfectants and offers knowledge that will enhance our capability to understand resistance mechanism of bacterial spores, allowing the design of novel control strategies. For example, studying treatments aiming at weakening the coat integrity or disrupt disulfide bonds may allow a decrease in the intensity of heat treatments required to ensure food safety and stability, better preserving quality or improving disinfection effectiveness,

avoiding (re) contamination.

CRediT authorship contribution statement

Víctor Freire: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Irene Orera:** Writing – review & editing, Methodology, Investigation. **Santiago Condón:** Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization. **Elisa Gayán:** Supervision, Methodology, Funding acquisition, Conceptualization.

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.

Acknowledgements

This study was supported by a grant [PID2019-104712RA-I00] from MCIN/AEI/10.13039/501100011033, and a P.h.D. scholarship from the Government of Aragón (to V.F.). The authors would also like to acknowledge the "Servicio General de Apoyo a la Investigacion-SAI" (University of Zaragoza) in developing the automatic colony counting system and Dr. C. Gross and the National BIO-Resource Project (NBRP) for providing us with the BKE genome-scale deletion library. We would like to express our sincere gratitude to Professor Santiago Condón Usón †, whose insightful discussions and contributions greatly enriched this work. Professor Santiago Condón Usón passed away during the preparation of this manuscript. We honor his memory and scientific legacy.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2025.117248.

Data availability

Data will be made available on request.

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