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## Quantifying the effect of sorbic acid, heat and combination of both on germination and outgrowth of *Bacillus subtilis* spores at single cell resolution



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### ABSTRACT

*Bacillus subtilis* spores are a problem for the food industry as they are able to survive preservation processes. The spores often reside in food products, where their inherent protection against various stress treatments causes food spoilage. Sorbic acid is widely used as a weak acid preservative in the food industry. Its effect on spore germination and outgrowth in a combined, 'hurdle', preservation setting has gained limited attention. Therefore, the effects of mild sorbic acid (3 mM), heat-treatment (85 °C for 10 min) and a combination of both mild stresses on germination and outgrowth of *B. subtilis* 1A700 spores were analysed at single spore level. The heat-treatment of the spore population resulted in a germination efficiency of 46.8% and an outgrowth efficiency of 32.9%. In the presence of sorbic acid (3 mM), the germination and outgrowth efficiency was 93.3% and 80.4% respectively whereas the combined heat and sorbic acid stress led to germination and outgrowth efficiencies of 52.7% and 27.0% respectively. The heat treatment clearly primarily affected the germination process, while sorbic acid affected the outgrowth and generation time. In addition a new 'burst' time-point was defined as the time-point at which the spore coat visibly breaks and/or is shed. The combined stresses had a synergistic effect on the time of the end of germination to the burst time-point, increasing both the mean and its variation more than either of the single stresses did.

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### 1. Introduction

Spore-forming bacteria are a problem for the food industry as they may survive many food preservation processes. Their spores can remain in a dormant, stress resistant state for a long period of time. Spores originate from soil and are ubiquitously present in fresh vegetables, milk as well as ingredients for processed foods such as herbs and spices or dairy products. Clearly, their inherent protection against various antimicrobial treatments poses

problems for microbiological food stability. The possible germination of such bacterial spores present in food products eventually causes food spoilage. Microbiological stability of food products is therefore highly dependent on the successful inactivation of bacterial spores. To that end the food industry is in search of innovative preservation techniques, which produce microbiologically safe, stable, nutritious, tasty and economically affordable foods. Complete thermal inactivation of microorganisms in food products has become less popular, primarily due to the negative effects on food quality and flavour (Hornstra et al., 2009; Leistner et al., 1995). Combinations of different in themselves mostly sub-lethal preservation treatments (hurdles) are thus used in order to achieve multitarget, often synergistic, effects leading to milder, but still fully reliable, food preservation strategies. Weak organic acids, such as sorbic acid and acetic acid, are commonly used preservatives and are often combined in such preservation approaches with thermal treatments (Ter Beek and Brul, 2010). These milder preservation

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treatments tend to fulfil customer demands whilst ensuring microbiological stability. In order to expand their scope, insight into effects at single cell level is highly desirable as genetically homogeneous populations often respond heterogeneously to treatments (see for instance Smelt et al., 2008 and Pandey et al., 2013). Such data are very important for the development of optimized predictions of bacterial growth in food products. Sorbic acid can cause a decrease in cytosolic pH and therefore affect normal cellular function (Ter Beek and Brul, 2010). This provides a mechanistic hypothesis for the effects seen in vegetative cells. At the population level van Melis et al. (2011) showed that at higher concentrations of sorbic acid, a reduction in the drop of OD<sub>600nm</sub> was seen for *Bacillus cereus* spores germinating in the presence of the weak organic acid versus those germinating in its absence. Hence this suggests a delay in germination caused by this weak acids food preservative. Here we set out to analyse the action of sorbic acid on spore germination and outgrowth in more detail.

It is known that dry and wet heat induces damage via different mechanisms. On the one hand dry heat primarily causes DNA damage (Setlow, 2006), whereas on the other hand wet heat causes protein denaturation in a nonspecific way, as was found using Raman spectroscopic techniques (Coleman et al., 2007). It was shown by Pandey et al. (2013) that heat treatment delays time to start of germination and increases the germination time, i.e. the time from phase bright to phase dark. However, there was no effect on both outgrowth and generation time of vegetative cells that emerged from the spores.

Prediction of bacterial growth can be complicated by the natural heterogeneity that bacteria exhibit, especially if they are present in low numbers. This heterogeneity occurs for example in the lag time between introduction of the spores to the germinants and the moment germination is initiated. This lag time can have great variation and is for example dependent on which germinant is used and at what concentration (Zhang et al., 2010). Germination time itself is largely dependent on the amount of germination receptors present in the inner membrane of the spore. On average, spores with higher levels of germination receptors germinate faster, though this is not the primary cause for heterogeneity observed in the time to start of germination (Zhang et al., 2013). Stringer et al. (2005) described the analysis of the time to start of germination and subsequent outgrowth of *Clostridium botulinum* using single cell microscopy. Previous research found that heterogeneity in spore germination and outgrowth could be increased by treatment with common preservation strategies. While, for example, a mild heat shock reduces heterogeneity in lag times of *Bacillus subtilis* spores (Smelt et al., 2008), more severe heat treatments increase heterogeneity and average duration of the time to start of germination both in *B. subtilis* and *C. botulinum* (Smelt et al., 2008; Stringer et al., 2011). The outgrowth can also be quite heterogeneously distributed. The heterogeneity observed for the different germination and outgrowth phases of *C. botulinum* was not correlated. The authors concluded that it is not possible to use time to start of germination as a prediction for the duration of later phases (Stringer et al., 2005).

At the population level, increased heterogeneity was observed in the outgrowth phase of *B. cereus* spores when they were exposed to 0.75 mM undissociated sorbic acid (den Besten et al., 2012). This was tested by a time resolved measurement of the change in optical density over time in microtiter plates. However, measurements of lag-time in a microtiter plate encompass the germination, outgrowth and vegetative growth phase. Therefore such measurements do not provide detailed information about neither variation within each of these phases, nor about heterogeneity in behaviour of individual spores within the population.

In this study, the three different stresses i.e. a mild heat stress

(85 °C for 10 min), sorbic acid stress (3 mM) and a combination of both mild stresses heat (85 °C for 10 min) and sorbic acid (3 mM) were studied for their effect on the different stages of spore germination and outgrowth. We probed for synergistic effects and, in addition to the previously defined phases of time to germination, germination time and outgrowth, we paid particular attention to the outgrowth phase in order to assess whether we could identify the emergence time-point of spores from their coat and cortex shell.

## 2. Materials and methods

### 2.1. Strain, media, and germination conditions

Spores of *B. subtilis* 168 laboratory wild-type strain 1A700 (*trpC2*) were used throughout the study. The spore crops were prepared in a defined minimal medium buffered with 3-(N-morpholino) propanesulfonic acid (MOPS) to pH 7.4 and harvested as described before (Kort et al., 2005). After harvest spores were stored in distilled water at 4 °C. The stored crops typically contained more than 99.9% of phase-bright spores. The harvested spores were first heat-activated in distilled water for 30 min at 70 °C. For sorbic acid stress experiments (end-concentration: 3 mM potassium sorbate), the heat-activated spores were subsequently allowed to germinate on defined minimal medium buffered with 80 mM of 2-(N-morpholino) ethanesulfonic acid (MES) at pH 6.4, supplemented with 10 mM L-asparagine, 10 mM D-glucose, 1 mM D-fructose and 1 mM potassium chloride (AGFK) as germinants. The experiments were performed in the closed-air containing chamber described by Pandey et al. (2013). For heat stress, the spores were heat-treated using the screw-cap tube method described by Kort et al. (2005). The heat-treated spore suspension was pelleted down at 4000 rpm for 15 min at 4 °C and resuspended in sterile water. These heat-treated spores were then (out)grown in defined minimal medium. For combined stresses, the spores were first heat-treated after heat-activation and then the thermally treated spores were allowed to germinate and grow out in sorbic acid containing minimal medium.

### 2.2. Microscope-slide preparation and time-lapse microscopy

A closed air-containing chamber developed by Pandey et al. (2013) was used for phase-contrast image acquisition. In brief, a cast was prepared by attaching a Gene Frame<sup>®</sup> to a standard microscope slide and cover slip. A thin, semisolid matrix pad of 1% agarose-solidified defined minimal (MES-buffered) medium (pH 6.4), supplemented with AGFK, loaded with heat-activated and/or heat treated spores, was mounted on a cover slip. Then the cover slip (containing the pad) was placed upside down onto the Gene Frame<sup>®</sup>. The resulting chamber was used for time-lapse microscopy (Pandey et al., 2013). The following conditions were tested: control (no stress), sorbic acid (3 mM), heat (85 °C for 10 min) and combination of both stresses. Two biological replicates and at least four technical replicates were performed for each stress. Maximally 9 different fields of view were recorded in parallel per experiment. Phase-contrast time-lapse series were recorded at a sample frequency of one frame per min for 8 h for control and sorbic acid stress, whereas 15 h observation time was chosen for heat stress experiments. This resulted in the analysis of approximately 70–100 spores from the start of each imaging experiment.

### 2.3. Data analysis with SporeTracker

To follow the germination and outgrowth process, and subsequent cell division in time, the decrease in pixel intensity and

increase in surface area were analysed, respectively. These parameters were measured using the image analysis tool “SporeTracker”, <<http://simon.bio.uva.nl/objectj/examples/sporetracker/SporeTracker.htm>>. SporeTracker is configured to measure different stages of germination and outgrowth as well as generation time of vegetative cells emerging from the spores in any desired time frame (Pandey et al., 2013). The different stages of development from dormant spores to dividing vegetative cells in the presence of stress (sorbic acid, heat-treated spore and combination of both) were compared with those of spores germinated in control condition. Differences in variance were tested with F-tests. Depending on the results of the F-tests the appropriate t-test or Welch's t-test (t-test with unequal variances) was performed to test differences in the average values. Germination and outgrowth efficiencies were tested with the  $\chi^2$  test. For all statistical tests, a significance level of 0.01 was used.

### 3. Results

The effect of sorbic acid, heat and a combination of both stresses was assessed on germination and outgrowth of *B. subtilis* 1A700 spores. The spores were allowed to germinate and grow out on agarose pads containing defined minimal medium buffered with MES at pH 6.4. For heat stress, the spores were heated at 85 °C for 10 min in a glycerol bath and were mounted on the developed cast. For sorbic acid stress as well as the combined stress exposure, the heat-activated and heat-treated spores respectively, were mounted on the developed cast with the agarose pad containing the required

amount of sorbic acid. Of all incubations the spore germination and outgrowth profile was next followed over time. Fig. 1 shows the still images of five different time points within a time frame of 8 h for the control (only defined minimal medium buffered with MES at pH 6.4), sorbic acid (3 mM), heat (85 °C for 10 min) and combination of both stresses (Supplementary movies S1, S2, S3, S4). Qualitatively the result can be summarized as follows. In the control incubations most spores had already germinated after 120 min. After 240 min, the spores had already progressed through the outgrowth phase and vegetative growth phase had begun. In the presence of 3 mM sorbic acid stress, the germination process was similar to germination under control condition, after 120 min most spores had germinated. However after 480 min, the micro colonies of cells were much smaller than in the control condition. This indicates the sorbic acid has an effect on outgrowth and/or vegetative cells. For heat stress, it is clearly observed that after 240 min a high number of spores are phase bright, which indicate the effect of heat on germination. This is similar for the combined stresses, the spores stay phase bright for a longer period of time, but after 480 min the micro colonies are smaller as well.

Supplementary video related to this article can be found at <http://dx.doi.org/10.1016/j.fm.2015.06.007>.

#### 3.1. Quantitative analysis of germination and outgrowth efficiency

The effect of different stresses on the germination and outgrowth of *B. subtilis* 1A700 spores was assessed by SporeTracker. The germination and outgrowth efficiency, i.e. germinated, non-

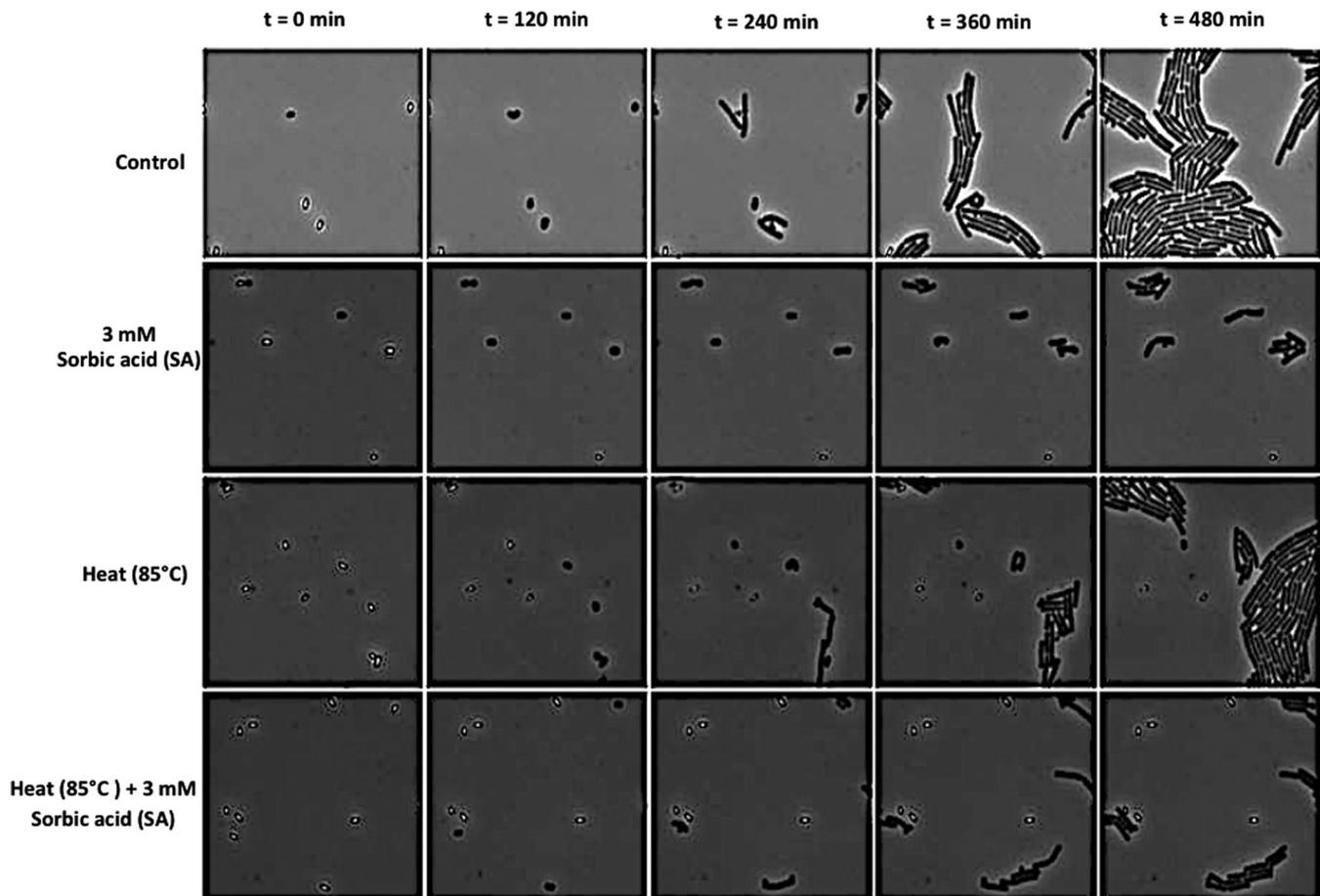


Fig. 1. Time-resolved images showing heterogeneous germination and outgrowth of *B. subtilis* 1A700 spores in control and stress conditions. Heat-activated and/or heat-treated spores (85 °C for 10 min) were spotted on defined minimal (MOPS-buffered) medium with or without sorbic acid and followed in time using phase-contrast microscopy.

germinated spores and germinated and outgrown as well as germinated but non-outgrown spores were compared for the control and different stress treatments. The heat-activated spores were either directly or after the described thermal inactivation treatment mounted on the developed cast with or without sorbic acid in the agarose pad and followed for germination and outgrowth behaviour over time (see Fig. 2 and Supplementary movies S1, S2, S3, S4). Fig. 2 shows the germination and outgrowth efficiency of un-treated spores (control), sorbic acid, heat and both stresses combined on spores. Of the spores exposed to control conditions, the germination and outgrowth efficiency is 94.1% and 89.2% (83.3% of total) respectively. For spores germinating in the presence of sorbic acid (3 mM) stress the germination and outgrowth efficiency is 93.3% and 87.1% (80.4% of total) respectively. This means that in the control ~4.9%, and 3 mM sorbic acid stressed incubations ~6.2% of the spores did not grow out. Instead, these spores stayed phase dark until the end of the movie. In heat-treated spores, the germination efficiency is 46.8% however the outgrowth efficiency is 86.1% (32.9% of total). Thus there is a significant decrease in particular in the germination efficiency as compared to the control. In combined stress (heat treated spores grown in 3 mM sorbic acid) the germination efficiency is 52.7%, which is similar to the heat-treated spores. But the outgrowth efficiency has here also dropped to 74.3% (27.0% of total), which is clearly lower than the heat-treated spores. Generally, the heat treatment (85 °C for 10 min) greatly reduced the germination efficiency of *B. subtilis* spores. Weak acid stress in the form of sorbic acid (3 mM) had no influence on both germination and outgrowth efficiency, however heat-treated spores germinating in the presence of sorbic acid (3 mM) showed a clearly decreased outgrowth efficiency compared to spores exposed to the 85 °C 10 min heat treatment alone. This points to a synergistic effect of sorbic acid and heat.

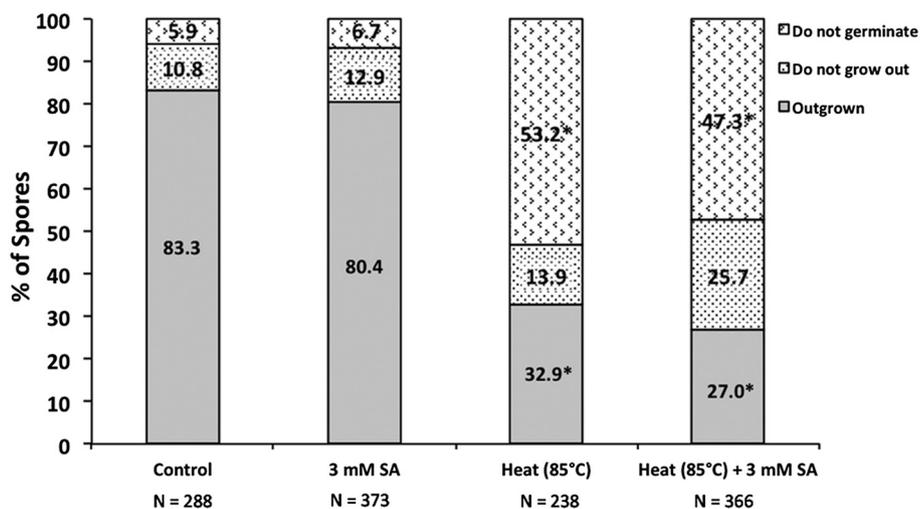
### 3.2. Germination heterogeneity

In phase contrast microscopy, spore germination is divided in two phases, the time to start of germination and the germination time. The time to start of germination was characterized by the time when the spore starts to become phase dark. The germination time is defined as the time required by the spore to transform from phase bright to phase dark. This occurs by the rapid release of

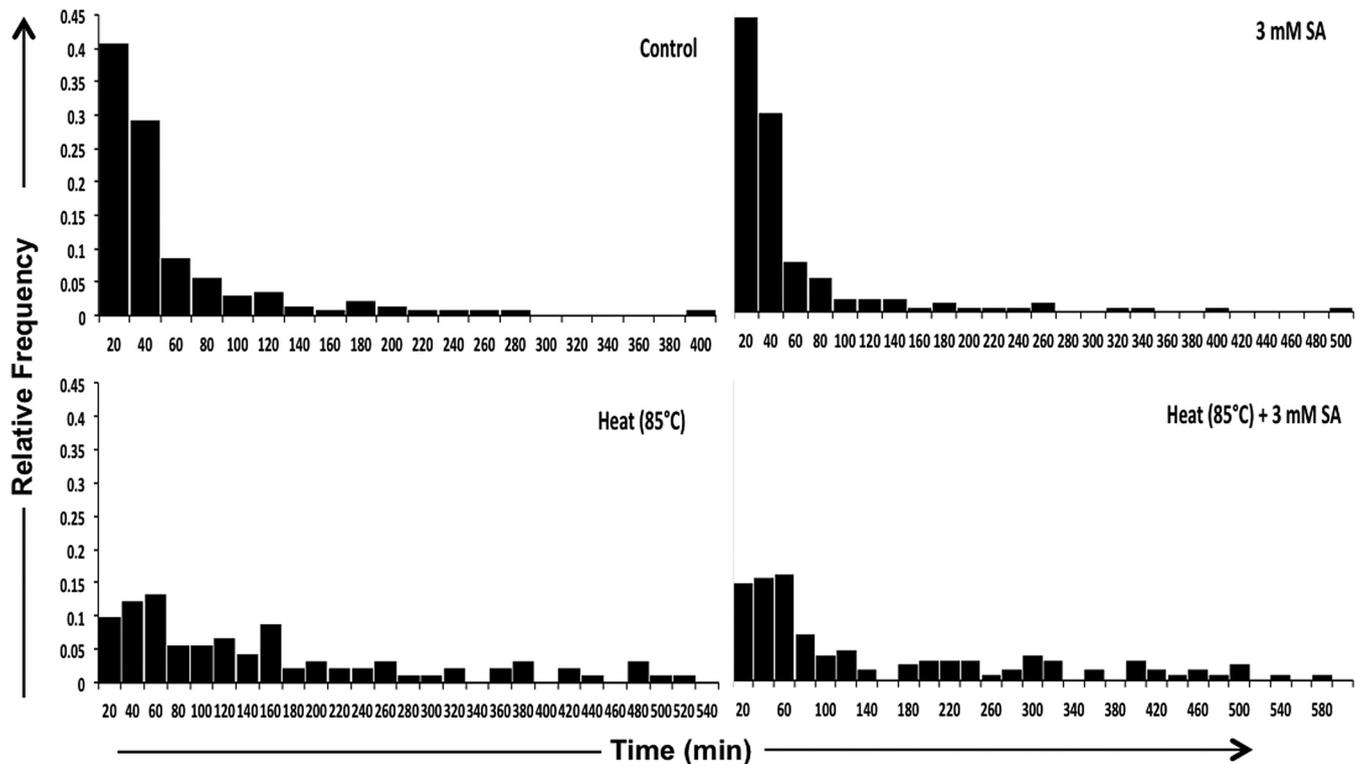
CaDPA and uptake of water, which causes a steep decrease in the spore's phase contrast intensity (e.g. Kong et al., 2010). In our study we could not analyse the germination time for all spores as at the first point of image acquisition 50% of the spores had already germinated during the time required for sample preparation. Fig. 3 shows the time to start of germination of the spores when spores were subjected to sorbic acid stress. Both the mean and variance of the time to start of germination are unaffected. In heat stressed spores, the mean is affected but not the variance whereas upon exposure to combined stress, the mean and variance both are affected (Table 1). Fig. 4 shows the effect of different stresses on the germination time of the spores. As observed previously (Pandey et al., 2013), the heat treatment (85 °C for 10 min) significantly delayed the germination time by about half a minute (Fig. 4B; Table 1). In the presence of sorbic acid (3 mM) an, albeit minor, trend towards faster germination was observed (Fig. 4B, Table 1). The variance in germination time of the heat and sorbic acid treated spore population is not significantly different from the control. The spores subjected to the combined stress have a significantly wider variation than the 3 mM sorbic acid stressed spores (Fig. 4B). In conclusion, the germination phases of the spores were primarily affected by the heat treatment as the heat stressed spores took longer time to initiate germination and the generation time itself is delayed by half a minute. Sorbic acid has no effect on both the time to start of germination and germination time.

### 3.3. Outgrowth heterogeneity

The phase between the end of germination and the first cell division is termed as outgrowth. In this phase the spore restarts its metabolism, repairs DNA damage and resume vegetative growth (see e.g. Keijsers et al., 2007; Sinai et al., 2015). The heat treatment had no effect on spore's outgrowth. In heat stressed spores, the mean outgrowth time was unaffected while its variation was increased. A small number of cells are characterized by a longer outgrowth time. Sorbic acid (3 mM) significantly increased the mean and variation of the outgrowth phase but not significantly the final numerical outcome in terms of growing colonies emerging from treated spores (Fig. 5, Table 1). The combined stress (heat and 3 mM sorbic acid) showed a strong effect on outgrowth as it significantly broadened the distribution (population



**Fig. 2.** Quantitative analysis of germination and outgrowth efficiency of heat, sorbic acid and combined stress on spores. Movies of un-treated, heat-treated (85 °C for 10 min), sorbic acid (3 mM) stressed and combined stressed spores were analysed with SporeTracker and the spores were scored for their ability to germinate and grow out. The total number of spores assessed in the control and stress condition was 288 (control), 373 (3 mM SA-treated spores), 238 (heat-treated spores), and 366 (heat and 3 mM sorbic acid-treated spores) respectively. Asterisks indicate a significance difference ( $\chi^2$ ,  $p < 0.01$ ) between the stress and control experiment.



**Fig. 3.** Analysis of individual spores with SporeTracker of the time to start of germination in the presence of sorbic acid, heat stress and the combined stress treatments. Movies of un-treated, heat-treated (85 °C for 10 min), sorbic acid and combined stressed spores (see Fig. 1 for details) were analyzed with SporeTracker. Frequency distributions of control, sorbic acid, heat and combination of both stresses on spores were calculated and are shown in black bars. The total number of spores assessed in the control, heat-treated spore, sorbic acid, and combination of both the stress were 223, 99, 285 and 146 respectively. The time resolution of the bins is 20 min and the first bin also contains those spores that had already germinated before the first observation time point.

heterogeneity). During the outgrowth phase the shedding of the spore coat and emergence of vegetative cells is observed. This process requires the hydrolysis of the spore cortex peptidoglycan by cortex lytic enzymes and the breakdown of the spore coat (Paredes-Sabja et al., 2011). Hence, the outgrowth time was further sub-divided in to two different phases. First, the time from the end of germination to the burst time and secondly the time from the spore coat burst to the first cell division. All stresses prolonged the time of end of germination to the time point of spore burst. Sorbic acid (3 mM) and heat stress (85 °C for 10 min) prolonged the mean duration, while both the stresses had no effect on the variation

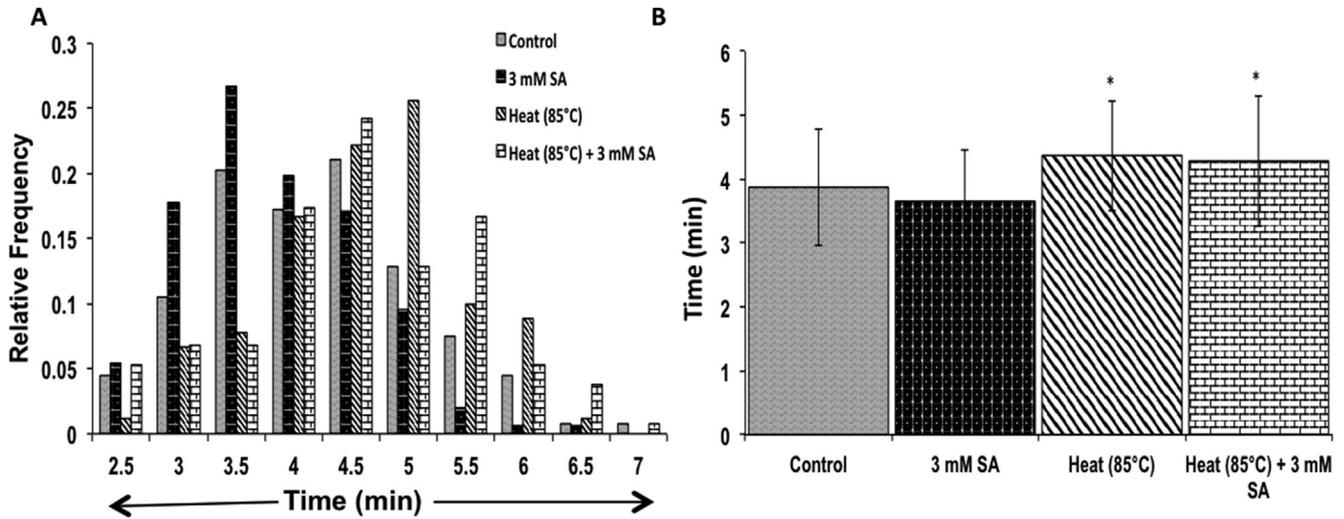
(Table 1). The combined stresses had a synergistic effect on the time of the end of germination to the burst time, increasing both the mean and its variation more than either of the stresses on their own (Table 1). This differs for the time of burst time to the first cell division. Here sorbic acid (3 mM) and the combined sorbic acid and heat stress increased the time of spore burst to the first cell division (Table 1). All stresses caused significant increase in the variation of burst time to the first cell division. It should be noted that an increase in time from the end of germination to the burst time and the time from the burst time to the first cell division under sorbic acid stress might have been expected, because they are both part of

**Table 1**  
Mean values  $\pm$  coefficient of variation (CV) of different stages of germination and outgrowth of individual heat activated *B. subtilis* spores with or without a pre heat-treatment (85 °C/10 min) and in the presence or absence of sorbic acid (3 mM).<sup>a</sup>

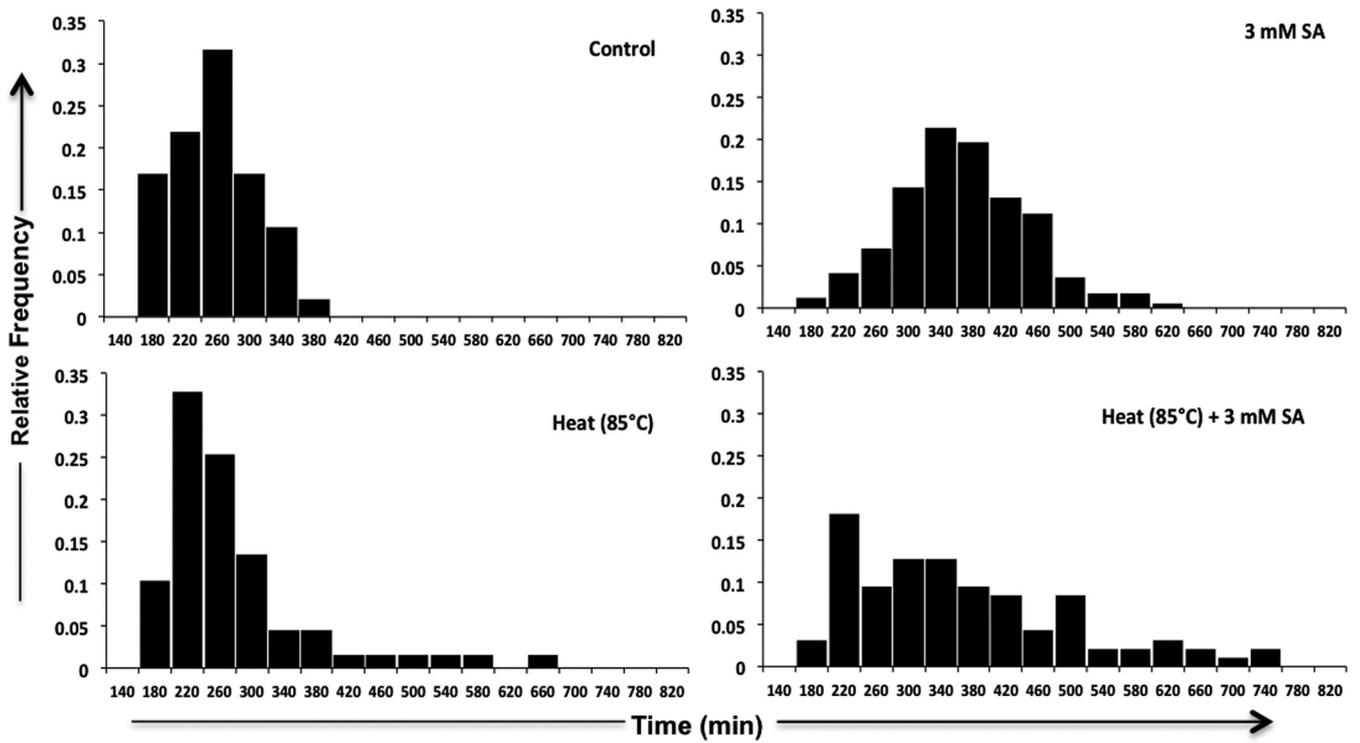
Mean $\pm$ CV <sup>b</sup>	Treatments			
	None	Sorbic acid (3 mM)	Heat-treatment (85 °C/10 min)	Sorbic acid (3 mM) + heat-treatment (85 °C/10 min)
Time to start of germination (min)	48 $\pm$ 1.2 (n = 140)	45 $\pm$ 1.4 (n = 162)	166 $\pm$ 1.0 (n = 91)*	169 $\pm$ 1.2 (n = 131)*†
Germination time (min)	3.9 $\pm$ 0.2 (n = 140)	3.7 $\pm$ 0.2 (n = 153)	4.4 $\pm$ 0.2 (n = 90)*	4.3 $\pm$ 0.2 (n = 132)*
Time of outgrowth (min)	236 $\pm$ 0.2 (n = 142)	350 $\pm$ 0.2 (n = 168)*†	259 $\pm$ 0.4 (n = 67)†	345 $\pm$ 0.4 (n = 94)*†
End of germination-	81 $\pm$ 0.4 (n = 107)	113 $\pm$ 0.4 (n = 79)*	97 $\pm$ 0.4 (n = 56)*	155 $\pm$ 0.5 (n = 70)*†
Burst time (min)				
Burst time-first cell division (min)	158 $\pm$ 0.2 (n = 107)	218 $\pm$ 0.3 (n = 79)*†	155 $\pm$ 0.4 (n = 56)†	192 $\pm$ 0.4 (n = 70)*†
Generation time (min)	52 $\pm$ 0.1 (n = 91)	88 $\pm$ 0.2 (n = 178)*†	54 $\pm$ 0.2 (n = 35)†	86 $\pm$ 0.2 (n = 48)*†

<sup>a</sup> Spores of *B. subtilis* 1A700 were heat-activated before heat-treatment and germination in defined minimal (MOPS-buffered) medium in the presence and absence of sorbic acid. Various germination and outgrowth parameters of individual spores were calculated as described in the materials and methods section.

<sup>b</sup> Mean time of different stages is given including the coefficient of variation. The amount of spores analysed from each stage are gathered from 2 to 5 (control, sorbic acid, heat and combination of both) independent biological replicates, which is given in brackets. The daggers indicate that the variance of the distributions between the stress and control experiment are significantly different (F-test,  $P < 0.01$ ). The asterisk indicates that the mean of the distributions between the stress and control experiment are significantly different (t-test,  $P < 0.01$ ).



**Fig. 4.** Analysis of individual spores with SporeTracker of the germination time of bacteria treated with heat, sorbic acid and both combined stresses. Movies of un-treated, heat-treated (85 °C for 10 min), 3 mM sorbic acid stressed and spores exposed to a combined stress (see Fig. 1 for details) were analysed with SporeTracker. Frequency distributions and average of germination time of bacteria in control, sorbic acid, heat and combination of both stresses were calculated and are shown in A and B, respectively. Asterisks indicate a significant difference ( $P < 0.001$ ) between the stress and control experiment.



**Fig. 5.** Analysis of individual spores with SporeTracker of spore outgrowth upon treatment with heat, sorbic acid and combined stresses. Movies of un-treated, 3 mM sorbic acid, heat treated (85 °C for 10 min) and spores subjected to a combined treatment (see Fig. 1 for details) were analysed with SporeTracker. Frequency distributions of their outgrowth time were calculated and are shown in black bars. The total number of spores assessed was 142, 67, 168 and 94 respectively.

the outgrowth phase which was overall prolonged with sorbic acid (3 mM) stress. An interesting observation in the control condition is that there is no correlation between the time from the end of germination to the burst time and the time from the burst time to the first cell division (Data not shown;  $R^2 = 0.046$ ). Thus, an increase in either of the phases does not automatically have to lead to an increase in the time of outgrowth. The small but significant increase in time from the end of germination to the burst time by heat stress could therefore be masked by shorter durations of burst time

to the first cell division and cannot be seen in a longer outgrowth duration. In conclusion, the sorbic acid (3 mM) stress has increased mean and variation of time of outgrowth. Heat stress increased the mean of the time of outgrowth but showed an increased effect on the variation. Similarly, as compared to sorbic acid (3 mM) the combined stress did not alter the mean time of outgrowth, but the variation has increased within the population. A new reference point in outgrowth was identified as the burst time of spores from their coat. All stresses increase the time from the end of

germination to the burst time, with a synergistic effect between heat (85 °C for 10 min) and sorbic acid (3 mM). The effect of the different stresses on the duration from the burst time–point to the first cell division shows that only 3 mM sorbic acid caused a significant increase in the time of this phase suggesting the key involvement of a lowered intracellular pH interferes in this phase with a normal resumption of cellular metabolic functions.

### 3.4. Generation time heterogeneity

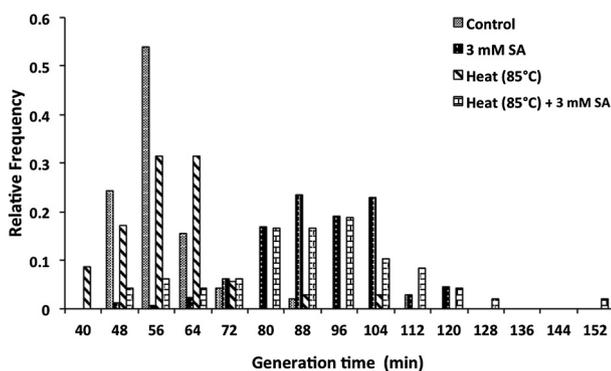
After the outgrowth phase the cell resumes normal growth. SporeTracker calculates the generation time by measuring the increase in surface area of the micro colonies. The spores in control conditions showed a generation time of  $52 \pm 0.1$  min. Heat stress did not increase the mean of the generation time significantly (Fig. 6, Table 1) however its variation was rendered significantly different. In the presence of sorbic acid (3 mM) and under a combined stress regime, the generation time was significantly increased by 50% with a difference in both its mean and variation (Fig. 6; Table 1). The shapes of the distributions of the generation time of the control cells and cells subjected to a heat treatment were very similar as were those of the sorbic acid (3 mM) exposed spores and the spores subjected to a combined (heat and sorbic acid) stress. Both 3 mM sorbic acid and the combined stress showed an increase in the average generation time.

## 4. Discussion

In this study phase contrast microscopy was used to analyse the effect of sorbic acid and/or heat stress on the germination, outgrowth and vegetative growth of *B. subtilis* spores at single spore level. This method allowed us to assess the impact of the stress on the various phases of spore ‘awakening’ i.e. germination and outgrowth phases. We thus analysed the time to start of germination, the germination time, the outgrowth time, which contained the time from the end of germination until the newly defined burst time and the time from spore burst until the first cell division, as well as, finally, the generation time. In addition to determining the average timing we could measure the level of heterogeneity for each and probed for possible combined (synergistic) effects of heat and sorbic acid stress. Heat treatment (85 °C for 10 min) largely affected the germination phases of *B. subtilis* spores. In accordance with Pandey et al. (2013) the thermal stress reduced the germination and outgrowth efficiency whereas it

increased the time to start of germination and the germination time. Sorbic acid (3 mM) mostly affected the outgrowth and generation time of emerging vegetative cells, increasing both the mean duration and the heterogeneity in these phases. At the molecular level, germination is a three steps process. Adequate levels of functional proteins are a prerequisite in the spore (Setlow, 2003). The first step is the recognition of germinants by their corresponding germination receptors. The second step is the release of CaDPA, for which the SpoVA protein is needed. This process starts with an initial slow release of CaDPA, thereby activating cortex lytic enzyme CwlJ, which is sensitive to either exogenous or endogenous CaDPA. Hydrolysis of cortex peptidoglycan by CwlJ in turn triggers fast CaDPA release and a drop in spore optical density intensity ( $OD_{600nm}$ ) (Kong et al., 2010; Paredes-Sabja et al., 2011). Third is the further hydrolysis of the cortex by cortex lytic enzymes CwlJ and/or SleB (Setlow, 2003).

Heat may well induce the denaturation of (some of) these proteins needed for germination. In this way the thermal treatment with wet-heat may cause both a reduction in germination efficiency (i.e. overall number of germinating spores within the time frame of the experiment) and for those spores that do germinate in the time to their germination start. First of all, heat treatment could reduce the amount of functional germination receptors in the inner membrane. This would lead to an increase in the time to start of germination (Stewart et al., 2012; Zhang et al., 2013; Smelt et al., 2008; Wang et al., 2011c). Due to the random aspect of heat inactivation, an increase in heterogeneity of the observed times to start of germination might also emerge. Interestingly, in our study, such increase in heterogeneity in the heat-treated spore population was not observed. The reason for this might be technical rather than biological. Cells growing out of heat-treated spores grow normally. This means that over the duration of 15 h often the field of view was completely overgrown with vegetative cells, making it impossible to determine the germination of a lot of late germinating spores. This makes the distribution appear more homogeneous. Therefore, for better analyses of the effect of heat treatment on the time to germination over longer time periods one could include for instance chloramphenicol in the medium to stop outgrowth and further vegetative growth. The antibiotic has no effect on the germination event itself (Smelt et al., 2008). The increase in germination time could also be explained by increased denaturation of either the SpoVA protein, which is an important component of the CaDPA channels or the cortex lytic enzyme CwlJ. Previously it was shown that the overexpression of SpoVA did not decrease the time required for the fast drop in optical intensity under phase contrast microscopy due to CaDPA release (Wang et al., 2011c). However, a decrease in duration of the initial slower CaDPA release was observed, thus decreasing the overall germination time. In the current study no distinction was made between different parts of germination as described in previous publications of the combined groups of Setlow and Li (Zhang et al., 2010; Wang et al., 2011c, 2011b), but germination was considered to be the overall time between the steady phase bright intensity before and phase dark after germination initiation. It could therefore very well be possible that a decrease in functional SpoVA could reduce the overall germination time and thus be in line with these observations. Finally it was previously shown that spores without the gene coding for the spore cortex lytic enzyme CwlJ have a slower germination phase as well (Wang et al., 2011c). Therefore, reduction of functional SpoVA and CwlJ caused by heat-treatment could account for the increase in germination time. In our study, the sorbic acid (3 mM) stress influenced the duration of the outgrowth phase and generation time but had no effect on germination or outgrowth efficiency. This is in contrast with previous results published by van Melis et al. (van Melis et al., 2011). At the



**Fig. 6.** Analysis of individual spores with SporeTracker of the generation time of cells subjected to heat, sorbic acid and combined stress regimes. Movies of un-treated heat-treated (85 °C for 10 min), 3 mM sorbic acid stressed spores and spores subjected to a dual stress regime (see Fig. 1 for details) were analysed with SporeTracker. Frequency distributions of the generation times measured were calculated and are shown with four different bars.

population level, van Melis et al. showed that at higher concentrations of sorbic acid, a slower drop in OD<sub>600nm</sub> was seen after introduction of *B. cereus* spores to germinants. Thus, van Melis et al. concluded that sorbic acid has an effect on *B. cereus* germination. It should be noted though that they used higher concentrations of protonated sorbic acid (HSA) than was used here. den Besten et al. (2012) showed an increase in heterogeneity in time until detection in microtiter plates with *B. cereus* upon germination in the presence of 0.75 mM HSA. Given our observations this increase in heterogeneity was likely caused by the effect of sorbic acid on (out)growing cells, and not by any effect on the germination itself.

In most cases, the germination or outgrowth phase was affected by either of the treatments while exposing cells to a combined stress regime generally showed the combined effect of both treatments. For example, the combination of heat and sorbic acid (3 mM) increased the average and the variance of the duration of the outgrowth phase. This was not significantly different from the increased time of outgrowth caused by the sorbic acid (3 mM) stress on the one hand and the increase in variance caused by only the heat treatment on the other hand (Table 1, Fig. 5). Though heat treatment increased the absolute variation compared to control and the combined treatment increased the absolute variation compared to the sorbic acid (3 mM) stress, in both cases the coefficient of variation was increased with a similar factor. Therefore synergy is not very obvious. A true indication for synergy would have been for instance an extra increase in outgrowth duration in the combination treatment or an extra increase in its heterogeneity. For three of the germination and (out)growth phases it was found that the coefficient of variation was similarly increased by the heat and combined treatments. This was the case for the outgrowth time, the time of burst until the first cell division and the generation time of vegetative cells. All three have in common that they were primarily affected by sorbic acid (Table 1). Synergism between heat and sorbic acid was found in the data on outgrowth efficiency (Fig. 2). It was observed for the time from the end of germination to the spore burst time. Here the additional presence of sorbic acid decreased the percentage of outgrown spores more than heat-treatment alone. In both control and sorbic acid stressed conditions about 6% of the germinated spores did not grow out. The possible options could be a failure in initiating early outgrowth processes (Keijser et al., 2007) or breakdown of the spore coat, thereby preventing elongation. Transcriptome analysis of germinating and outgrowing spores has shown that processes related to outgrowth take place at a slower rate under sorbic acid stress (van Melis et al., 2011). No mechanistic data is available about the effect of heat on early outgrowth processes, though the initial increase could be explained by the cell repair damage received during heat treatment. Because sorbic acid causes early outgrowth processes to slow down, it can be expected that the repair of heat induced damage under sorbic acid stress would take longer as well. However, the data presented here does not give clear information about whether the weak acid stress causes the cell to cope worse with heat induced damage, or whether heat damage causes the cell to cope worse with the weak organic acid preservative stress. It is likely that both of the synergistic effects observed here are intertwined, as both decreased outgrowth efficiency and increased the time from the end of germination to the spore burst time point indicating that the cell has trouble with the initiation and execution of early outgrowth processes.

To gain more insight in the 'dynamic range' of the stresses used in this study, increasing concentrations of sorbic acid, stress temperatures, thermal treatment times or combinations of heat and weak organic acid stress should be studied to see in what measure the effects seen here increase with increased stress levels. Spores in food products are under constant stress of a combination of

different preservative systems such as weak acids i.e. acetic acid, sorbic acid and other/or preservatives. It is therefore very relevant to quantify the effects of a preservation regime in which a physical (thermal) stress is combined with preservative stresses at the level of single spores with respect to their germination and outgrowth behaviour. This provides relevant information about the heterogeneity in different phases of germination and outgrowth of spores, which leads to a better understanding of the behaviour of bacterial spore formers and should steer the development of a growth model relevant to food products. An important parameter is a GFP resolved measurement of internal pH as direct method to investigate whether heat treatment causes the outgrowing cells to cope worse with sorbic acid stress. This can be done with fluorescence microscopy using a pH sensitive green fluorescent protein called pHluorin (Miesenböck et al., 1998). Expression of this protein in the spore allows ratiometric pH<sub>i</sub> assessment thus facilitating a time resolved measurement of the internal pH during different stages of germination and outgrowth of spores exposed to various (combined) environmental stress conditions.

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### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.fm.2015.06.007>.

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