**ORIGINAL PAPER**

# Characterization of individual spores of two biological insecticides, *Bacillus thuringiensis* and *Lysinibacillus sphaericus*, in response to glutaraldehyde using single-cell optical approaches

Huanjun Chen[1] · Xiaochun Wang[1] · Cuimei Li[1] · Xiaoling Xu[2] · Guiwen Wang[1]

## Abstract

*Bacillus thuringiensis* (Bt) and *Lysinibacillus sphaericus* (Ls) are the most widely used microbial insecticides. Both encounter unfavorable environmental factors and pesticides in the field. Here, the responses of Bt and Ls spores to glutaraldehyde were characterized using Raman spectroscopy and differential interference contrast imaging at the single-cell level. Bt spores were more sensitive to glutaraldehyde than Ls spores under prolonged exposure: <1.0% of Bt spores were viable after 10 min of 0.5% (v/v) glutaraldehyde treatment, compared to ~20% of Ls spores. The Raman spectra of glutaraldehyde-treated Bt and Ls spores were almost identical to those of untreated spores; however, the germination process of individual spores was significantly altered. The time to onset of germination, the period of rapid $Ca^{2+}$–2,6-pyridinedicarboxylic acid (CaDPA) release, and the period of cortex hydrolysis of treated Bt spores were significantly longer than those of untreated spores, with dodecylamine germination being particularly affected. Similarly, the germination of treated Ls spores was significantly prolonged, although the prolongation was less than that of Bt spores. Although the interiors of Bt and Ls spores were undamaged and CaDPA did not leak, proteins and structures involved in spore germination could be severely damaged, resulting in slower and significantly prolonged germination. This study provides insights into the impact of glutaraldehyde on bacterial spores at the single cell level and the variability in spore response to glutaraldehyde across species and populations.

**Keywords** Raman spectroscopy · Differential interference contrast imaging · Spore germination · Glutaraldehyde · *Bacillus thuringiensis* · *Lysinibacillus sphaericus* · Single-cell analysis

## Introduction

*Bacillus thuringiensis* (Bt) and *Lysinibacillus sphaericus* (Ls), both spore-producing bacilli, are two of the most widely used microbial insecticides (Guidi et al. 2013; Zhang et al. 2023). Bt is the most widely produced and used live microbial pesticide worldwide, especially for green and organic food production. Bt spore formation is accompanied by the production of specific proteins, which lepidopteran larvae ingest and are then converted into toxic peptides by the action of proteases within the insect midgut's alkaline environment. Toxic peptides bind specific receptors on the epithelial cell membrane of the larval intestine, causing membrane perforation and leading to general paralysis, spasm, and death in the insect (Azizoglu et al. 2023; Crickmore 2006). Ls is a safe and effective biocontrol agent for mosquitoes. Ls forms protein crystals consisting of binary toxins (BinA and BinB proteins), along with spore formation, which serve as its primary virulence factor targeting *Culex* spp. and *Anopheles* spp. larvae (Ahsan and Shimizu 2021; Hernandez-Santana et al. 2016; Hu et al. 2008). Although the crystal protein is the primary function of Bt and Ls pesticides, it is worth noting that Bt spores, either on their own or in conjunction with crystals, can also have toxic

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✉ Guiwen Wang
  wguiwen@gxas.cn

[1] Institute of Eco-Environmental Research, Guangxi Academy of Sciences, 98 Daling Road, Nanning, Guangxi 530007, China

[2] Agriculture and Food Engineering College, Baise University, Baise, Guangxi 533000, China

effects on insects (Crickmore 2006) and even increase virulence (Bulla et al. 1975). Ls spores, meanwhile, are capable of producing virulence proteins in deceased larvae under certain circumstances. However, both Bt and Ls formulations are living microbial preparations and may encounter unfavorable conditions, such as aldehyde pesticides, which affect their activity and therefore spore germination and cell growth, as well as recirculation in infected insects, which in turn affects the biocontrol effect and persistence of insecticides.

Bacterial spores are dormant bodies that form in vegetative cells when bacteria are exposed to unfavorable growth conditions. Spores are resistant to extreme conditions because of their extremely low water content and are rich in $Ca^{2+}$–2,6-pyridinedicarboxylic acid (CaDPA, ~ 10% dry weight) (Leggett et al. 2012; Setlow 2006, 2014b). Bacterial spores are commonly inactivated by radiation (UV, gamma radiation, etc.), thermal treatment (dry heat, moist heat, etc., alone or in combination with ultrahigh pressure), and chemical agents (Liang et al. 2023; McDonnell and Russell 1999; Pérez-Díaz et al. 2023; Russell 1990). Glutaraldehyde is an important chemical disinfectant with high efficiency, low toxicity, wide range of applications, and stable performance. Glutaraldehyde alkylates the carboxyl, hydroxyl, amino, and sulfhydryl groups of proteins, causing protein coagulation and the death of microorganisms (Lasemi et al. 2017; Retta and Sagripanti 2008; Russell 1994). Studies on *B. subtilis* spores have shown that glutaraldehyde does not damage spore DNA, but the treated spores are unable to grow into vegetative cells (Tennen et al. 2000). Glutaraldehyde probably inactivates germinant receptors (GRs) and blocks spore germination (Power and Russell 1990a, b), or affects the outer membrane, possibly the spore cortex, forming a dense outer shell and preventing germination (Tennen et al. 2000). Previous studies have only been conducted at the population level, and details at the single-cell level remain unclear. Studies on the effects of glutaraldehyde on Bt and Ls spores are limited. Additionally, the viability and reproduction of Bt and Ls formulations after application are crucial factors in assessing the environmental safety and efficacy of biological agents. However, knowledge of the response of Bt and Ls spores to external factors is limited. Therefore, it is crucial to recognize the response of biocides to environmental factors, particularly glutaraldehyde, at the single-cell level. This recognition has significant implications for the development of novel biopesticides and insect disease control.

Optical technology approaches, including Raman spectroscopy, differential interference contrast (DIC) microscopy, fluorescence imaging, and Raman imaging combined with single-cell analysis have played an important role in life science research (Cordero et al. 2018; Laine et al. 2016; Maxmen 2011; Puig and Kaltenbach 2018). Raman spectroscopy is insensitive to water and requires no special sample preparation; Raman signals are specific, providing rich information about the molecular structure and composition, and therefore, they are widely used in biomedical fields (Casabella et al. 2016; Cordero et al. 2018; Ember et al. 2017; Germond et al. 2017; Smith et al. 2016). Single-cell analysis based on Raman spectroscopy is an exciting and rapidly expanding field for understanding complex biological systems at the cellular level (Chan 2013; Jayan et al. 2022). Multicellular real-time DIC microscopy allows high-throughput, real-time monitoring of physiological processes and dynamic activities of a large number of several individual cells at the single-cell level, which has greatly contributed to our understanding of cell physiology and the roles of biotic and abiotic factors (Chen et al. 2015; Qiu et al. 2023; Setlow 2013, 2014a; Sundaresan and Cheong 2024; Wang et al. 2011b; Xing and Harper Jr 2020). Therefore, in this study, we used single-cell optical approaches to monitor the response of Bt and Ls spores to glutaraldehyde and investigated the effects of glutaraldehyde on spore structure and spore germination-related proteins. We found that Ls spores were more sensitive to glutaraldehyde than Bt spores at short exposure times, but showed more resistance to glutaraldehyde at long exposure times. Glutaraldehyde may not only have damaged spore GRs, but also inactivated cortex lytic enzymes (CLEs) and CaDPA release channels. Glutaraldehyde may have severely affected the proteins associated with important stages of germination in both Bt and Ls spores. These findings provide insights into the mechanism of glutaraldehyde-mediated killing of bacterial spores at the single-cell level as well as the heterogeneity of the response to glutaraldehyde in spores from different species or within the same population.

## Materials and methods

### Experimental strains and spore preparation

*B. thuringiensis* HD-1 was purchased from the China General Microbiological Culture Collection Center (AS 1.1014). *L. sphaericus* 2362 was kindly provided by Dr. Xiaomin Hu (Wuhan Institute of Virology, Chinese Academy of Sciences).

Stock cultures of the Bt and Ls strains were grown on Luria–Bertani pH 7.4–7.6 (LB; peptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L) plates for 24–36 h. A single colony was transferred to liquid LB medium and incubated overnight with shaking at 200 rpm. Then, 1% Bt broth was transferred to sporulation medium (CCY) (Stewart et al. 1981) and incubated for 48–60 h with shaking at 200 rpm, and 2%

Ls broth was transferred to NYSM medium (g/L; nutrient broth 19, yeast extract 0.5, $MnCl_2 \cdot 4H_2O$ 0.009895, $CaCl_2$ 0.0777, $MgCl_2 \cdot 6H_2O$ 0.2033) and incubated for 48–60 h with shaking at 250 rpm. The incubation temperature was set at 30 °C. The spores were collected by centrifugation at $3000 \times g$ and washed 10 times with sterile water. Purified spores were free (99%) of growing and sporulating cells, germinated spores, and cell debris, as verified by phase-contrast microscopy and stored at 4 °C.

## Glutaraldehyde treatment

Glutaraldehyde (0.2% and 0.5%; v/v) was prepared in 0.3% (w/v) $NaHCO_3$, and 2.0% (w/v; pH 6.0) glycine was prepared in sterile water. Next, 0.2 mL of spore suspension ($OD_{600} = 10.0$) was centrifuged in a 2-mL centrifuge tube at $3000 \times g$ for 6 min to remove the supernatant. The spores were incubated with 0.2% (or 0.5%) glutaraldehyde (0.2 mL) at 25 °C for the time needed, and then treated with 1.8 mL of 2.0% glycine for 10 min to inactivate glutaraldehyde. The supernatant was removed by centrifugation at $3000 \times g$ for 6 min, washed twice with sterile deionized water, resuspended in 0.2 mL sterile deionized water, and stored at 4 °C. Untreated spores were used as controls.

## Measurement of spore viability

The treated and untreated spores were serially diluted to obtain $10^{-3}$- to $10^{-6}$- fold gradients. Then 0.1 mL of the spore solution of each gradient was evenly spread on LB Petri dishes. Each gradient was repeated three times. The plates were incubated at 30 °C for 24–36 h, and the number of colonies formed on LB agar was counted. Spore viability was calculated as the number of colonies that grew after glutaraldehyde treatment. Untreated spores were used as controls.

## Raman setup and measurement

Raman spectroscopy was setup as described (Xie et al. 2002). Briefly, a 780 nm laser beam was introduced into an inverted microscope (TE2000-U, Nikon) and focused by a 100× objective (N.A. = 1.30) to form an optical trap that captured spores suspended in water. Raman scattering excited by the same laser beam was collected with the same objective and focused on a spectrometer (LS785, Acton), and imaged on a CCD (PIXIS 400BR, Princeton Instruments, Inc.). Polystyrene microspheres (2.0-µm diameter) were used to calibrate the system. Then 0.2 mL of treated or untreated spore suspension was loaded into a homemade chamber. Individual spores were randomly trapped by the laser beam and Raman

spectra were acquired with an acquisition time of 20 s. Spectra acquired without spores in the trap were used as the background. In total, 50 spectra and 5 background spectra were collected for each treatment.

The acquired spectra of individual spores were subtracted from the background prior to 5-point adjacent smoothing and baseline correction using polynomial fitting. The total area under the peaks at 783, 1017, and 1650–1670 $cm^{-1}$ were calculated and used as the signal intensity of these peaks.

## Germination of individual spores

In total, $10^8$ spores were suspended in 1 mL of sterile water and heat-shocked in a water bath (70 °C for Bt, 80 °C for Ls) for 30 min and then put in an ice bath at 0 °C for 15 min. Bt spores were germinated under the following conditions: (i) 50 mmol/L L-alanine in 25 mmol/L HEPES buffer (pH 7.4) at 37 °C, (ii) 45 mmol/L CaDPA at 37 °C, and (iii) 0.8 mmol/L dodecylamine in 25 mmol/L HEPES buffer (pH 8.3) at 45 °C. Spores with CaDPA- and dodecylamine-triggered germination were not heat-shocked.

Ls spores were triggered by (i) 50 mmol/L L-alanine in 25 mmol/L HEPES buffer (pH 7.4) at 30 °C; (ii) 60 mmol/L CaDPA at 30 °C; and (iii) 0.8 mmol/L dodecylamine in 25 mmol/L HEPES buffer (pH 8.3) at 45 °C.

A DIC microscope (Nikon Ti-U) was equipped with a CCD, and the germination of individual spores was monitored in real time, as previously described (Chen et al. 2015). Spore images were acquired every 3–6 s, with an exposure time of 200 ms, for 120 min. The brightness of individual spores was read, imported into Origin 8.5, and plotted as a DIC brightness variation curve for germination versus time. Germination parameters were read as described in previous reports (Chen et al. 2015).

The release of CaDPA during spore germination involves two phases: slow and rapid release (Wang et al. 2015). Statistical analysis revealed that the rapid release phase of germnation lasted approximately 1 min and was similar across species and under different germinants (unpublished data). Differences between spores were observed mainly in the slow-release phase. Therefore, we considered the two phases of CaDPA release as one phase and defined the times of CaDPA release onset, CaDPA release completion, and completion of peptidoglycan cortex hydrolysis as $T_{lag}$, $T_{release}$, and $T_{lys}$, respectively. Thus, the time to complete the release of CaDPA was $\Delta T_{release} = T_{release} - T_{lag}$, and the time to complete peptidoglycan cortex hydrolysis was $\Delta T_{lys} = T_{lys} - T_{release}$.

The germination of treated Bt spores was extremely slow in response to dodecylamine, making it challenging

to determine the time of complete CaDPA release by DIC imaging. Therefore, population germination was monitored by measuring the $OD_{600}$ of spore cultures using a spectrophotometer, and germination of individual spores was monitored using single-cell Raman spectroscopy as described by (Zhang et al. 2018).

## Statistical analysis

The results for the average means and standard deviations of the germination parameters were compared to the untreated control using a one-way ANOVA. $P$-values of * ($p = 0.05$) and ** ($p = 0.01$) indicate statistical significance.

## Results

### Spore viability and Raman spectra of individual spores

Figure 1-a illustrates the viability of Bt and Ls spores after treatment with 0.2% or 0.5% glutaraldehyde for different periods of time. After exposure to 0.2% (v/v) glutaraldehyde for 5 ~ 30 min, the viability of Bt spores was 34% ~ 0.07%. When exposed to 0.5% (v/v) glutaraldehyde for the same duration, the viability of Bt spores decreased to approximately 20% ~ 0.06%. The viability of Ls spores subjected to 0.2% (v/v) glutaraldehyde treatment for 5 ~ 30 min was 34% ~ 16%. In contrast, spores exposed to 0.5% (v/v) glutaraldehyde for the same period showed a viability of approximately 26% ~ 0.7%. Ls spores showed increased sensitivity to glutaraldehyde during short exposures, but greater resistance during longer exposures.

The average Raman spectra of the Bt spores before and after 0.5% glutaraldehyde treatment are shown in Fig. 1-b. The peaks at 661, 824, 1017, 1397, 1449, and 1573 $cm^{-1}$ are characteristic of CaDPA. Peaks at 1220–1290 $cm^{-1}$ and 1630–1680 $cm^{-1}$ are assigned to the protein secondary structure, 1260–1300 $cm^{-1}$ and 1645–1655 $cm^{-1}$ to the protein α-helix secondary structure, and 1245–1270 $cm^{-1}$ and 1655–1665 $cm^{-1}$ to β-folding and irregular (random) folding (Puppels et al. 1990; Talaria et al. 2015). The average Raman spectra of Bt spores treated for 20–30 min were similar to those of untreated spores, and there were no significant differences in the characteristic peaks of protein secondary structure or in the Raman intensities of nucleic acids (783 $cm^{-1}$), CaDPA (1017 $cm^{-1}$), and proteins (1660 $cm^{-1}$) (Fig. 1-c). Similar results were obtained for the Ls spores treated in the same manner (data not shown).

## Effect of glutaraldehyde on the germination of individual spores

### Nutrient germination

Small nutrient molecules can awaken and germinate dormant spores. Figure 2-a shows Bt spore germination after treatment with 0.2% glutaraldehyde and induction with L-alanine. Untreated spores germinated more rapidly, with 90% of the spores germinating within 60 min. After glutaraldehyde treatment for 3, 5, and 10 min, the spore germination was approximately 76%, 52%, and 41%, respectively, after 120 min. The germination dynamics of individual spores showed that the time to initiate rapid release of CaDPA ($T_{lag}$), time to complete release of CaDPA ($\Delta T_{release}$), and time to complete hydrolysis of peptidoglycan cortex ($\Delta T_{lys}$) were significantly higher in treated spores (Fig. 2-b, -c). After 3, 5, and 10 min of treatment, the average $T_{lag}$ values of individual spores were increased approximately 1-, 3-, and 3.4-fold, $\Delta T_{release}$ values were increased 1.7-, 7-, and 27-fold, and $\Delta T_{lys}$ values were approximately 0.6-, 1-, and 1.6-fold higher than those of the control (Fig. 2-e). Notably, biphasic release of DPA was observed after 5 min of treatment (Fig. 2-c). Additionally, the relative brightness at $T_{lag}$ of the treated spores was significantly lower than that of the untreated spores (approximately 0.85) after 5–10 min of treatment. Extrapolating from the relative brightness (Wang et al. 2011), approximately 19% of CaDPA was released at $T_{lag}$, compared to approximately 6% in untreated spores. These findings are similar to those reported for moist heat-treated *B. subtilis* spores (Wang et al. 2011).

Inosine is a nutrient that triggers Bt spore germination (Fig. 2-a). Most untreated spores germinated within 10 min. Treatment with 0.2% glutaraldehyde for 3 and 5 min resulted in 38% and 16% germination at 120 min, respectively, whereas no spores germinated at 120 min after 10 min of treatment. The release of DPA was biphasic (Fig. 2-d). $T_{lag}$ values were approximately 2- and 3-fold higher, $\Delta T_{release}$ values were approximately 11- and 46-fold higher, and $\Delta T_{lys}$ values were approximately 1- and 1.3-fold higher than those of the control after 3 and 5 min of treatment, respectively (Fig. 2-f).

L-Alanine germination of Ls spores after treatment with the same concentration of glutaraldehyde showed that untreated spores germinated faster, with 94% of the spores germinating within 60 min; however, after 3–10 min of treatment, spore germination became significantly slower, and the germination rate decreased, with 74–19% spores germinating within 120 min (Supplementary Fig. S1-a). Similar to the germination of Bt spores in L-alanine, the $T_{lag}$, $\Delta T_{release}$, and $\Delta T_{lys}$ values of Ls spores after 3–10 min of treatment were 3.1–7.6-, 1.1–1.5-, and 0.6–0.7-fold higher

**Fig. 1** (**a**) Viability of *Bacillus thuringiensis* (Bt) and *Lysinibacillus sphaericus* (Ls) spores after treatment with 0.2% or 0.5% glutaraldehyde for different periods of time. (**b**) Average Raman spectra of individual Bt spores treated with 0.5% glutaraldehyde for 0, 20, and 30 min and difference spectra between 30 min and 0 min, (**c**) Raman intensities of peaks at 783, 1017, and 1660 $cm^{-1}$, shown as the mean and standard deviation
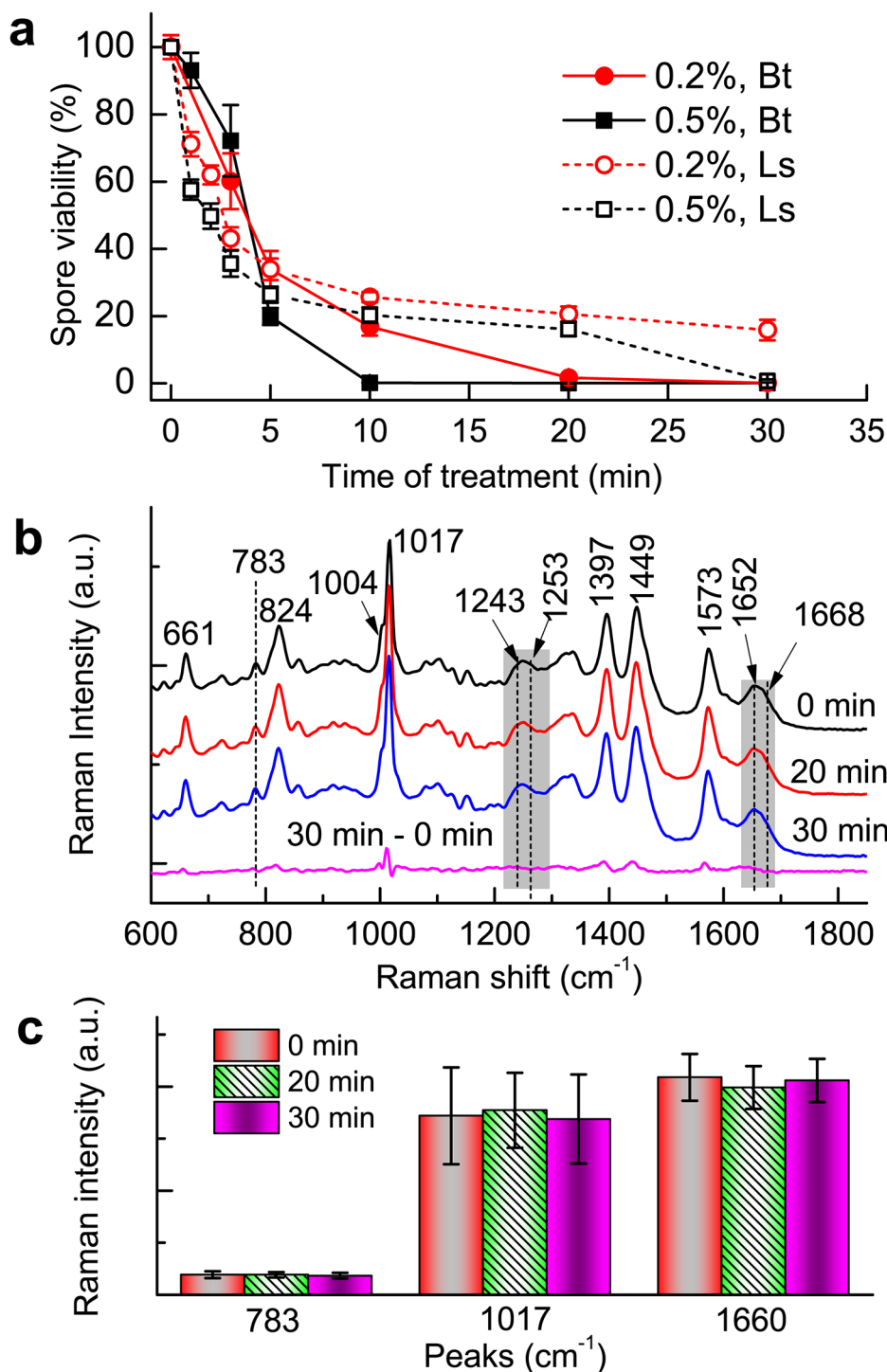


than those of untreated spores, respectively (Supplementary Fig. S1-b–d).

## Non-nutrient germination

Exogenous CaDPA induces germination by activating the cortical hydrolase (CwlJ) in spores' outer levels (Paidhungat

et al. 2001). Bt spores are extremely susceptible to exogenous CaDPA: >99% of untreated spores germinated completely within 10 min, spores treated for 3 min began to germinate only after 20 min of exogenous CaDPA stimulation, approximately 24% of the spores treated for 5 min germinated during the 120-min observation period, and
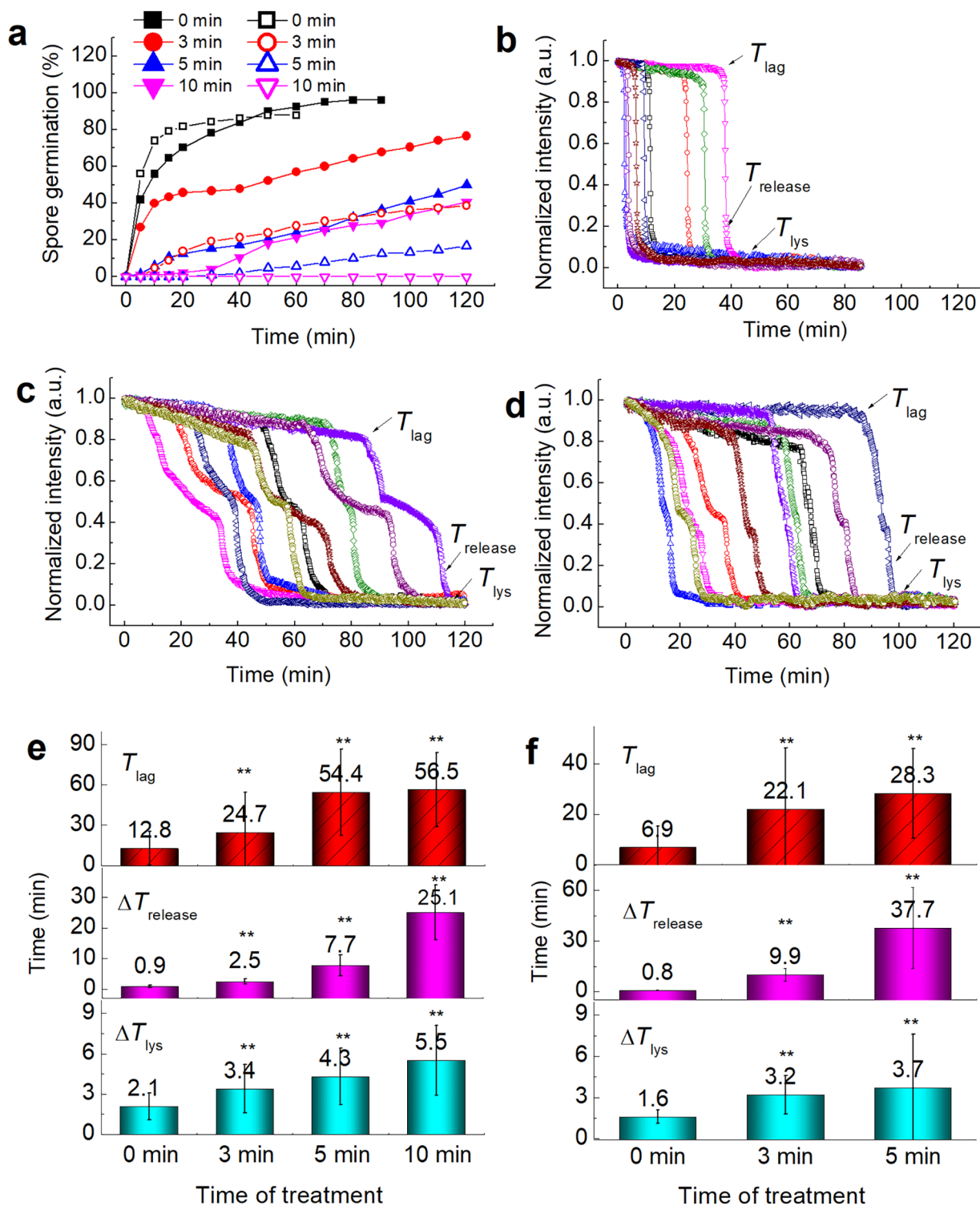
**Fig. 2** Germination of *Bacillus thuringiensis* spores after 0.2% glutaraldehyde treatment. Germination was induced with L-alanine or inosine and monitored as described in the Materials and Methods. (**a**) Spore germination (%) induced with L-alanine (closed symbols) or inosine (open symbols); (**b–d**) Germination dynamics of 10 individual spores, (**b**) untreated spores induced by L-alanine, (**c**) 5-min-treated spores induced by L-alanine, (**d**) 3-min-treated spores induced by inosine; (**e, f**) germination parameters induced by (**e**) L-alanine or (**f**) inosine, shown as the mean and standard deviation (** $p = 0.01$; $n = 50$)

spores lost their germination ability after 10 min of treatment (Fig. 3-a).

Untreated Ls spores began to germinate after 20 min of exogenous CaDPA challenge, and 97% of the spores germinated after 120 min. After treatment with 0.2% glutaraldehyde, most spores began to germinate after ~30 min. The germination percentages after 1, 2, 3, and 5 min of treatment were 89%, 81%, 76%, and 22%, respectively. Spores lost their germination ability after 10 min of treatment (Supplementary Fig. S2). The germination parameters of individual spores showed that the average $T_{lag}$, $\Delta T_{release}$, and $\Delta T_{lys}$ values of spores after 1, 2, and 3 min of treatment were 1.5–2.3-, 1–1.6-, and 1.1–1.2-fold those of the control, respectively.

## Dodecylamine germination

Dodecylamine is a cationic surfactant that induces non-nutrient germination of spores, presumably by opening the release channels on the inner spore membrane, leading to CaDPA release (Setlow et al. 2003). In contrast to nutrient germination, dodecylamine germination shows no obvious course of cortical hydrolysis (Tennen et al. 2000). Glutaraldehyde treatment severely affected dodecylamine-triggered germination of Bt and Ls spores. The germination percentages of Bt spores were 47.3% and 35% after 3 and 5 min of treatment, respectively, compared with 99% for untreated spores (Fig. 4). CaDPA release from treated Bt spores was extremely slow, resembling the nutrient germination of *B. subtilis* spores that lack CLEs (Peng et al. 2009). After 3 and 5 min of treatment, the average $T_{lag}$ and $\Delta T_{release}$ values were 0.75-, 1.5-, and 4-fold higher than those of the control, respectively.

Dodecylamine germination of untreated Ls spores was 95% within 120 min and 64%, 51%, and 30% after 3, 5, and 10 min of treatment with 0.2% glutaraldehyde, respectively (Supplementary Fig. S3). The $T_{lag}$ and $\Delta T_{release}$ values of treated individual spores increased significantly; after 3–10 min of treatment, the $T_{lag}$ and $\Delta T_{release}$ values were 3.1–5.7- and 2–3-fold higher, respectively, than those of untreated spores (Supplementary Fig. S3-d).
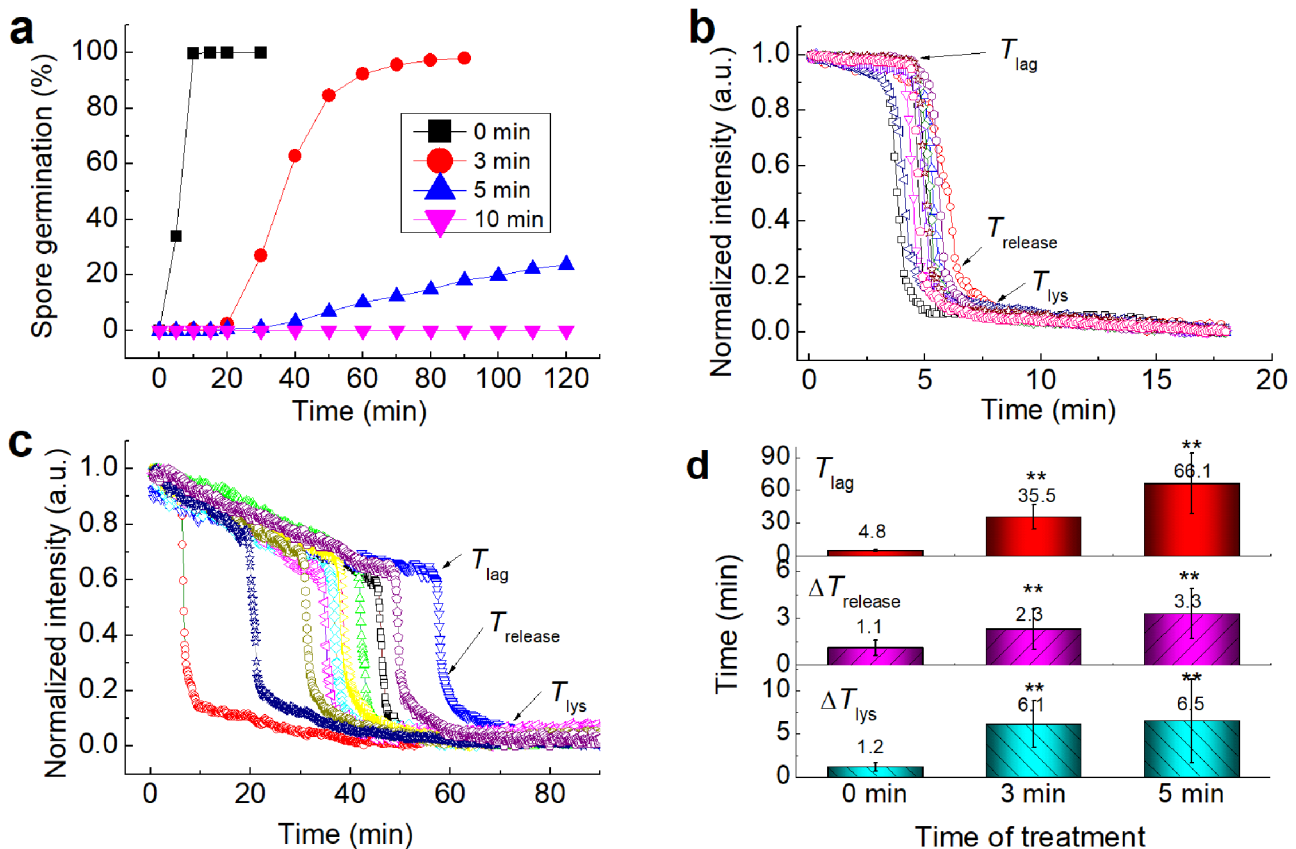


**Fig. 3** CaDPA germination of *Bacillus thuringiensis* spores after 0.2% glutaraldehyde treatment. Germination was triggered by exogenous CaDPA and followed as described in the Materials and Methods. (**a**) Spore germination (%); (**b, c**) germination dynamics of 10 individual (**b**) untreated or (**c**) 3-min-treated spores; (**d**) germination parameters of individual spores, $T_{lag}$, $\Delta T_{release}$ and $\Delta T_{lys}$, shown as the mean and standard deviation (** $p = 0.01$; $n = 50$)

**Fig. 4** Dodecylamine germination of *Bacillus thuringiensis* spores after 0.2% glutaraldehyde treatment. Germination was triggered by 0.8 mmol/L dodecylamine and followed by spectrophotometry and Raman tweezers as described in the Materials and Methods. (**a**) Spore germination (%); (**b, c**) germination dynamics of 6 individual (**b**) 3-min-treated or (**c**) untreated spores; (**d**) germination parameters of individual spores, $T_{lag}$, and $\Delta T_{release}$, shown as the mean and standard deviation (** $p = 0.0$; $n = 20$)
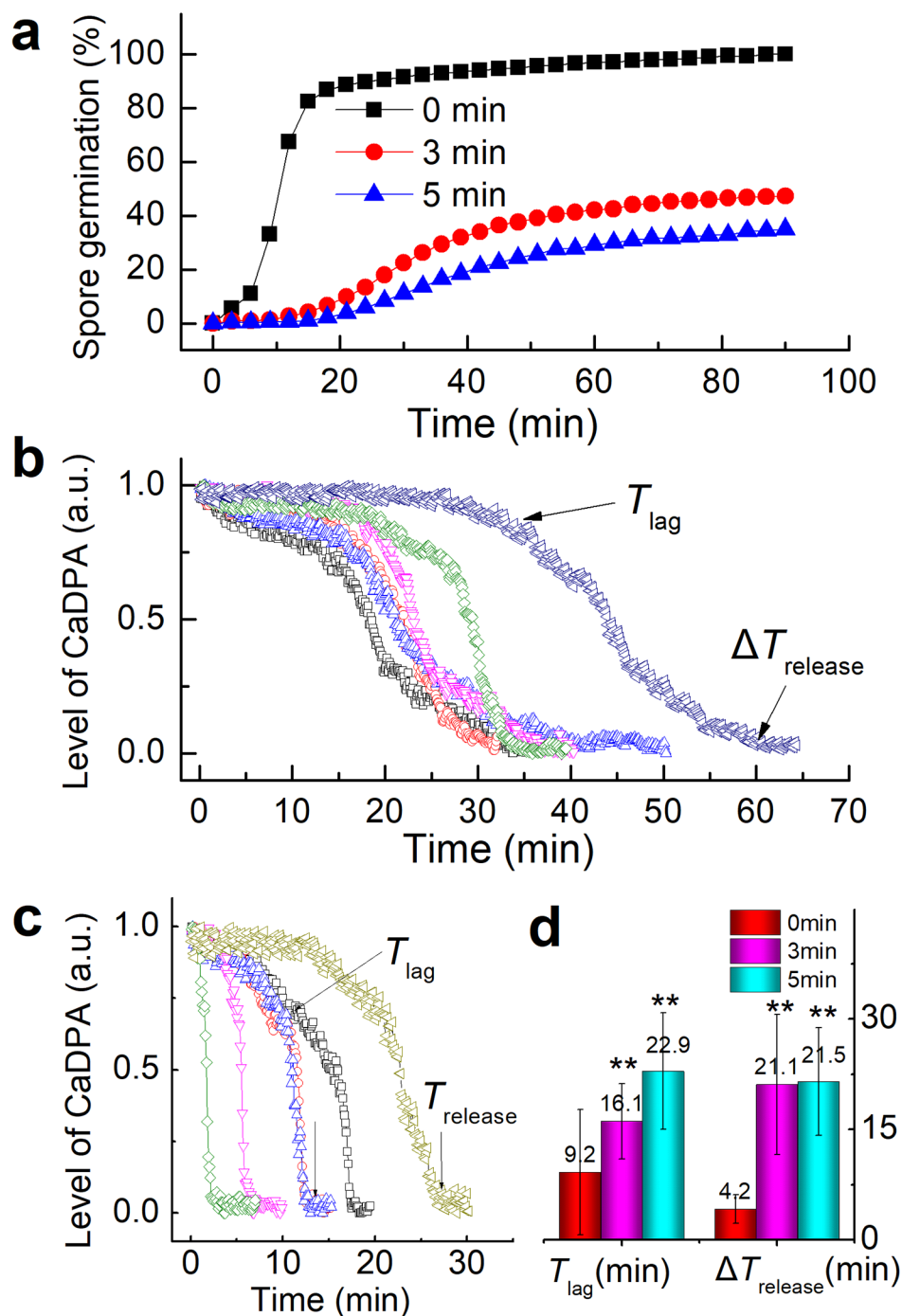
## Discussion

Bt is a widely used and successful microbial insecticide (Zhang et al. 2023). It is genetically close to *B. cereus* and *B. anthracis* and is the surrogate strain of *B. anthracis* for field release (Park et al. 2017). Recent studies have shown that Bt strains can potentially biodegrade residual pollutants in the environment. Additionally, Ls have a wide range of potential applications (Ahsan and Shimizu 2021; Jamal

and Ahmad 2022), such as plant growth promotion, bioremediation of heavy metal or chemical contamination, food preservation, biological disease control, wastewater treatment (Lin et al. 2023), and even mortar surface treatment and crack healing in the construction industry. Therefore, exploring the response of Bt and Ls spores to external factors is of practical significance.

The responses of *B. subtilis* spores to glutaraldehyde have been studied at the population level (Lasemi et al.

2017; Power and Russell 1990a, b; Tennen et al. 2000), but details at the single-cell level are unclear. Glutaraldehyde is an effective sporicidal agent, with spores of different species showing different responses. Only 0.2% of Bt spores were culturable after 10 min of treatment with 0.5% glutaraldehyde, whereas 20% of Ls spores were culturable, indicating that Ls spores are more tolerant to glutaraldehyde under prolonged exposure. However, even after 20 and 30 min of treatment, no obvious change was observed in the position and intensity of the characteristic peaks, particularly in the 1220–1290 cm$^{-1}$ and 1620–1680 cm$^{-1}$ regions, which are associated with the protein secondary structure (Fig. 1). This indicated that the effect of glutaraldehyde on Bt and Ls spores was significantly different from that of other disinfectants; glutaraldehyde did not break the spore barrier, and the main components of spores, such as CaDPA, proteins, and nucleic acids, did not leak.

Bacterial spores can be awakened by sensing extracellular nutrients, peptidoglycan fragments, or exogenous CaDPA, surfactants (dodecylamine), and high pressure (Setlow 2013; Xing and Harper Jr 2020). The binding of nutrients, such as L-alanine, inosine, and L-valine, triggers spore germination and initiates a series of events, including the release of monovalent cations ($H^+$, $K^+$, $Na^+$, $Zn^{2+}$, etc.) in the very early stages, as well as CaDPA, a key substance in the spore core (Gao et al. 2024; Moir 2023). This is followed by the hydrolysis of peptidoglycans in the spore cortex, water uptake by the spore core, and expansion of the cell wall. Proteins in the spore core gain mobility, enzymes become active, and the spores lose dormancy characteristics (Christie and Setlow 2020; Fan et al. 2023; Lyu et al. 2023; Setlow 2013). Exogenous CaDPA activates cortical hydrolases to hydrolyze the spore cortex, triggering the release of CaDPA from the spore core to the outside through channels in the inner pore membrane (Setlow 2013). Surfactants such as dodecylamine can induce spore germination by opening CaDPA release channels in the inner spore membrane, either directly or indirectly (Setlow et al. 2003). Several proteins are involved in germination, such as GRs and GerP, GerD proteins in nutrient sensing; related proteins in transducing the germination signal, channel proteins on the spore envelope, such as the SpoVA membrane complex, in regulating CaDPA release, and CLEs, such as SleB and CwlJ, as well as other ancillary proteins, such as those in the spore envelope, GerQ, and YpeB, which are involved in cortex degradation (Paredes-Sabja et al. 2011; Setlow 2014a; Xing and Harper Jr 2020). Receptor proteins are involved in nutrient germination, but not in germination induced by nonnutrient germinants such as exogenous CaDPA and dodecylamine. Impairment of the function of any of these protein components affects spore germination. Therefore, the response of spores to physical or chemical agents can be determined by analyzing the germination dynamics of individual spores under different germination conditions.

The $T_{lag}$ value of L-alanine or inosine for the germination of treated Bt spores was 1–3- or 3–4-fold higher than that of untreated spores (Fig. 2); for Ls spores, the corresponding value was 3.1– to 7.6-fold higher than that of untreated spores (Fig. S1). The onset of rapid CaDPA release (i.e., $T_{lag}$ values) is affected by heat activation, germinants, the number of GRs (Zhang et al. 2010), and related proteins (GerP, YlaJ, and YhcN) that contribute to germination. The number of GRs and related proteins that can sense the germinants may be a factor affecting the $T_{lag}$ value, since heat activation and germinant concentration were constant during nutrient germination. Therefore, we speculated that the $T_{lag}$ values of Bt and Ls spores increased because GRs and complementary proteins were partially damaged by glutaraldehyde, rendering them nonfunctional.

Upon receiving the germination signal, channels on the inner membrane open and CaDPA begins to rapidly release, a process that is independent of heat shock, number of GRs, and concentration of germinants (Setlow et al. 2006). The SpoVA membrane complex is crucial in CaDPA uptake during spore formation and in CaDPA release during spore germination (Gao et al. 2022, 2024; Vepachedu and Setlow 2007; Wang et al. 2011a). When the release channel is impaired, the release of CaDPA slows. The $\Delta T_{release}$ values of the nutrients (Fig. 2 and Fig. S1) and non-nutrient germination (Figs. 3 and 4, S2, and S3) significantly increased after glutaraldehyde treatment. In particular, in the nutrient germination of Bt spores, the average $\Delta T_{release}$ value of spores after 10 min of treatment increased 26-fold compared with that of the control. Biphasic release of CaDPA appeared with increasing treatment intensity (Fig. 2-c, -d). In dodecylamine germination, the $\Delta T_{release}$ values of Ls spores after 3–10 min of treatment increased significantly and were 2–3-fold higher than those of untreated spores (Fig. 4-d). These results suggest that glutaraldehyde severely affects the inner spore membrane, leading to the impairment of CaDPA release channels and related proteins.

Functionally impaired proteins may also incude CLEs, such as CwlJ, SleB, and the adjacent GerQ and YpeB. CLEs hydrolyze the spore cortex and expand the spore core via water uptake. Cortical hydrolysis causes the release of CaDPA from the core, which triggers spore germination (Paidhungat et al. 2001). When the activity or number of CLEs is reduced, cortex hydrolysis slows, and hydrolysis is prolonged. CaDPA germination, which depends on the action of CLEs, is greatly affected. The cortical hydrolysis time for nutrient germination for Bt and Ls spores ($\Delta T_{lys}$) was 1.6–2.6- and 1.6–1.7-fold that of the control, respectively, after 3–10 min of treatment (Fig. 2 and S1). In contrast, in exogenous CaDPA germination, the hydrolysis time

of Bt and Ls spores increased 4- and 0.2-fold, respectively, after 3 min of treatment (Fig. 3 and S2). Thus, glutaraldehyde caused more severe damage to the CLEs of Bt spores than to those of the Ls spores.

Inactivation of GRs by glutaraldehyde may inhibit *B. subtilis* spore germination (Power and Russell 1990a, b). However, by comparing the changes in the germination dynamics of individual spores before and after treatment, we speculate that glutaraldehyde may not only damage and inactivate spore GRs but also inactivate CLEs and CaDPA release channels and have severe effects on proteins associated with several important stages of Bt and Ls spore germination. Scanning electron microscopy revealed no obvious changes in the morphology after high-intensity glutaraldehyde treatment (data not shown). Comparison of germination data revealed that the culturable percentages of Bt spores treated with 0.2% (v/v) glutaraldehyde for 5 and 10 min were 34% and 17%, respectively (Fig. 1), which were significantly lower than those induced by L-alanine for the 120-min observation period (52% and 41%, respectively; Fig. 2), indicating that although some of the treated spores could germinate, they did not grow into vegetative cells. Similar results were obtained for the treated Ls spores (Fig. 1 and S1). This may be due to the cross-linking of glutaraldehyde with the spore envelope and cortex, which causes the spores to form a dense shell that prevents germinated spores from growing into vegetative cells and forming colonies (Tennen et al. 2000).

Microorganisms exhibit genetic and phenotypic heterogeneity, which is important for their survival (Ackermann 2015). There was considerable variability in the responses of Bt and Ls spores to glutaraldehyde. Ls spores were more responsive to glutaraldehyde at short exposures but showed greater resistance to glutaraldehyde at longer exposures (Fig. 1). The standard deviation of germination parameters among spores from the same population increased with the intensity of glutaraldehyde treatment (Fig. 2e, f ; Fig. S1-d; Fig. 3-d), indicating increased intercellular variation and significant differences in the responses of different spores to the same concentration of glutaraldehyde. Thus, Bt or Ls spores from the same population show significant heterogeneity in their resistance to disinfectants, and incomplete disinfection or inappropriate treatment may allow highly resistant individual spores to survive and reproduce.

## Conclusion

The response of spores to glutaraldehyde has been extensively examined at the population level. However, individual spore responses to glutaraldehyde have rarely been reported. This study used single-cell optical techniques to monitor the spore response to glutaraldehyde in two biopesticides. Our findings revealed noticeable variations in the response of Bt and Ls spores to glutaraldehyde. Ls spores showed increased sensitivity to glutaraldehyde during short exposure times but greater resistance during longer exposures. Analysis of single-cell Raman spectra showed no discernible impact on the internal architecture and composition of the spores. However, the germination dynamics of individual spores under nutrient and non-nutrient germinants revealed that glutaraldehyde may damage spore GRs and disrupt CLEs and CaDPA release channels. Aldehyde disinfectants should not be used on live microbial preparations as they can have serious effects on the proteins associated with important stages of germination in both Bt and Ls spores.

**Data availability** The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Competing interests** The authors declare no competing interests.

**Conflict of interest** The authors declare no conflicts of interest.

**Ethical approval and consent to participate** This study involved two spore-forming bacterial species, neither of which requires ethical approval.

**Consent for publication** All authors read and approved the manuscript.

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