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Insight into the inactivation of *Bacillus cereus* spores by ohmic heating and application in liquid food

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ABSTRACT

Bacillus cereus spores pose a potential threat to various food products, including meat, grain, vegetables, and dairy products, due to their remarkable resistance to environmental stress. Ohmic heating (OH) is an emerging alternative technology for inactivating spores. This study aimed to investigate the effects of OH on *B. cereus* spores, specifically examining its impact on heat resistance, leakage of intracellular substances, integrity of the cortex and membrane, and structural changes, in order to elucidate the potential inactivation mechanism of OH. The results demonstrated the efficacy of OH in inactivating *B. cereus* spores in various liquid food products while maintaining product quality. The wet heat resistance of spores decreased after OH treatments at low-temperatures (60–70 °C), potentially promoting subsequent inactivation. Conversely, resistance increased following OH treatments at higher temperatures and oil bath heating (OB) treatments. Similar inactivation effects were observed between OB and OH when the temperature reached 80 and 98 °C. Both OH and OB treatments damaged spore structures, resulting in leakage of intracellular substances and disruption of homeostasis. However, OH treatment at 10 V/cm and 50 Hz resulted in less leakage of dipicolinic acid, protein, nucleic acids within the spores, and appeared to have additional effects on the structures within the inner membrane. These findings could facilitate the development of OH as a promising technology for effective spore control in food.

1. Introduction

Bacillus cereus is a ubiquitous Gram-positive, spore-forming bacterium. Its ability to produce highly resistant spores is a critical factor in its capacity to contaminate food and cause illness. *B. cereus* spores can form during food process or enter the food supply chain through water, soil, and raw food materials, thereby contaminating meat, grain, vegetable, and dairy products[1-3]. Under favorable conditions, these spores germinate and proliferate, producing toxins that lead to food spoilage and severe foodborne illnesses[4]. Understanding the specific structure and properties of these spores is crucial for developing effective control measures. The exceptional resistance of spore to environmental stresses arises from its complex, multi-layered structure. From the outermost to the innermost, these layers include the exosporium, coat, outer membrane, cortex, germ cell wall, inner membrane, and core[5]. Each layer uniquely contributes to the spore's resilience. The spore coat is significant for resistance to certain chemicals and exogenous cortex-lytic enzymes[6]. The spore cortex is essential for maintaining low water content in the spore core and facilitating the formation of dormant spores[7]. The inner membrane, characterized by its low permeability, plays a major role in the spore's resistance to many chemicals[8]. Finally, the core's low water

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content, along with the presence of dipicolinic acid (DPA), and small, acid-soluble spore proteins (SASPs), provides additional protective effects[9]. Therefore, *B. cereus* spores are pathogenic and exhibit exceptional resistance to environmental stresses, posing a substantial challenge for the food industry. To address this issue, various technologies, such as radiation, cold plasma, high pressure, and heat have been employed to control *B. cereus* spores during food processing and preservation[10].

Among these techniques, thermal treatment remains one of the most effective methods for spore inactivation and is still the primary technology used in food manufacturing. Ohmic heating (OH), an innovative alternative thermal technology, heats food by converting electrical energy into thermal energy as electric current passes through the food[11]. This heating mechanism allows OH to generate heat rapidly and uniformly while preserving the nutritional and sensory qualities of food products. OH systems have gained popularity in the food industry due to their numerous advantages and have found commercial applications in the United States, Europe, Japan, and Mexico[12]. Furthermore, OH offers potential for inactivating foodborne pathogens by leveraging both thermal and electrical effects. It has demonstrated effectiveness against Gram-positive and Gram-negative vegetative bacteria[13-14], and bacterial spores[15]. OH can effectively inactivate *B. cereus* spores in thick and highly viscous sauces[16].

The primary mechanism of microorganism inactivation by OH is typically attributed to thermal effects[17]. However, evidence suggested the existence of additional nonthermal effects, as OH has been shown to achieve better inactivation results and inflict more severe damage to bacterial cells than conventional heating[18-19]. Tola et al.[20] reported that OH exhibited a marginally better inactivation rate of *Bacillus coagulans* spores and demonstrated the presence of additional non-thermal effects compared to conventional heating. Similarly, Singh et al.[21] found the OH with higher electrical field strength exhibited more effective killing of *Clostridium sporogenes* PA3679 spores in significantly shorter durations through a synergistic effect of electricity and temperature. While electroporation is often cited as the primary non-thermal effect of OH in the inactivation of vegetative bacterial cells, the inactivation of spores may involve more complex mechanisms due to their intricate structure. Schottroff et al.[15] confirmed an additional inactivation effect on *B. subtilis* spores during OH, with the primary target of the electric field being within the spore's core. The chemical composition and spore structure play an important role in spore resistance[9]. Although these studies have provided valuable insights into the inactivation effects of OH on spores, the exact mechanisms through which OH damages *B. cereus* spore structures remain limited, warranting further investigation.

Therefore, the primary objective of this study is to investigate the effectiveness and potential mechanism of controlling *B. cereus* spores using OH, with implications for improving food safety and preservation techniques. We evaluated the inactivation efficiency, heat resistance, release of the intracellular substances, and changes in spore structure during OH treatments to analyze and identify the targets of OH treatment on *B. cereus* spores. Additionally, we examined the inactivation efficiency, pH, color, and viscosity of milk, juice, soymilk, and broth to explore the application of OH in liquid food and provide an experimental foundation for future industrial application.

2. Materials and methods

2.1 Bacterial strain and spore preparation

B. cereus ATCC 11778 (Shanghai North Connaught Bio-Tech Co., Ltd., Shanghai, China) was streaked onto a nutrient agar plate. A single colony was subsequently inoculated into 50 mL of Brain-Heart Infusion broth (Qingdao Hope Bio-Technology Co., Ltd., Qingdao, China) and incubated overnight at 37 °C with continuous shaking at 200 r/min. *B. cereus* spores were prepared according to the method described by Lv et al.[22], with minor modifications. The *B. cereus* cells were sporulated on tryptic soy agar (Solarbio, Beijing, China) supplemented with 0.05 g/L $MnSO_4 \cdot 4H_2O$ (Macklin, Shanghai, China). The culture was incubated aerobically for 7 days at 37 °C to facilitate sporulation, followed by an additional 7 days at 30 °C to complete the lysis of sporulating cells. The sporulation rates of the spores used in this study were ≥ 95%. Spores were harvested and washed 6–8 times with sterile deionized water by centrifuging at 8 000 × *g* for 15 min at 4 °C (TGL-20M, Pingfan Instrument Co., Ltd., Changsha, China). Subsequently, the spores were resuspended in sterile deionized water at a concentration of approximately 1×10^9 CFU/mL and stored at 4 °C for future use within 15 days.

2.2 Heating treatments

2.2.1 Inactivation effects of B. cereus spores inoculated in PBS by OH and conventional heating treatments

To analysis the inactivation effects and structure changes of spores, a 1% inoculation with 1×10^9 CFU/mL of *B. cereus* spores in phosphate-buffered saline (PBS, 0.01 mol/L NaH_2PO_4, 0.03 mol/L Na_2HPO_4, and 0.09 mol/L NaCl, pH 7.20) (Sinopharm Group Co., Ltd., Shanghai, China) was prepared to obtain a spore suspension of 1×10^7 CFU/mL.

The specifications of the OH device are consistent with those detailed in our previous report[23]. The OH equipment includes a power supply system (0–160 V, 45–500 Hz), a heating unit, and a data acquisition system. A total of 160 mL of *B. cereus* spore suspension (1×10^7 CFU/mL) was placed in the heating unit and subjected to OH treatment at 10 V/cm, 50 Hz (OH-50Hz) and 10 V/cm, 500 Hz (OH-500Hz). The temperature and current were continuously recorded throughout the treatment. For the OH-50Hz treatment, the times required to reach the temperature of 60, 70, 80, 90, and 98 °C were 1.8, 2.1, 2.4, 2.8, and 3.2 min, respectively. In contrast, the OH-500Hz treatment required 4.9, 6.1, 7.3, 8.6, and 9.8 min to reach the same temperatures.

To enhance the heating rate, an oil bath (OB) was employed as the conventional heating method. The time required for spore suspension to reach 98 °C was controlled by adjusting the temperature of the OB. The inactivation efficiency of spores by the OB methods decreased with reduced time to reach 98 °C. An OB treatment time of 18.2 min (OB temperature of 125 °C) was selected to achieve comparable inactivation effects of *B. cereus* spores as observed with the OH treatments. A total of 160 mL *B. cereus* spore suspension (1×10^7 CFU/mL) was transferred into a high-temperature-resistant plastic cylindrical container (diameter 4.5 cm, length 10.0 cm, thickness 0.1 cm) and heated in a thermostatic OB of 125 °C. The spore suspension was consistently agitated, and the center temperature was recorded using a

thermometer throughout the heating process. The OB required 5.3, 7.6, 10.4, 14.1, and 18.2 min to reach the temperature of 60, 70, 80, 90, and 98 °C, respectively.

Spore samples from the center of the heating unit for both OH and OB treatments were extracted using sterile injectors when the preset center temperatures were reached during the heating process. These samples were then rapidly cooled to 4 °C on ice for subsequent analysis.

2.2.2 Inactivation effects of B. cereus spores inoculated in different liquid food

Pasteurized milk (Sanyuan Food Co., Ltd., Beijing, China), orange juice (Weiquan Food Co., Ltd., Hangzhou, China) and soy milk (containing 2.0 g/L brown rice flour, and 1 g/L oat flour, Dali Food Co., Ltd., Quanzhou, China) were obtained from the local supermarket. Nutrient broth (containing 10 g/L peptone, 5 g/L NaCl, and 3 g/L beef extract powder, Solarbio, Beijing, China) was autoclaved at 121 °C for 20 min. A 1% inoculum of 1×10^9 CFU/mL *B. cereus* spores was added to the milk, orange juice, soy milk, and broth to obtain a final spore concentration of 1×10^7 CFU/mL. The samples were then subjected to OH (10 V/cm, 50 Hz) and OB treatments with a final temperature of 98 °C.

2.3 Enumeration of viable spores and determination of heat resistance of spores

The viable number of spores was determined using the total plate count method. Samples were serially diluted in a 0.85% NaCl solution, and 100 μL of the appropriate dilutions were plated on nutrient agar. Plates were incubated at 37 °C for 12 to 24 h. The reduction in spore counts was calculated as the difference in the spore counts (lg (CFU/mL)) before and after OB and OH treatments.

The wet heat resistance of spores (80 °C) was determined according to a reported method with some modifications[24]. Untreated spore suspension (initial temperature was 25 °C) and spore suspensions treated with OB and OH to 60, 70, 80, 90, and 98 °C (as prepared in Sections 2.1 and 2.2) were subsequently heated in a water bath at 80 °C for 0–120 min. Spore count reduction was calculated as the difference in spore counts (lg (CFU/mL)) before and after wet heat treatment. D-values (decimal reduction times, min) at 80 °C were calculated from the negative reciprocal of the slope of the survivor curve's linear regression.

2.4 Determination of nucleic acids, proteins, and DPA leakage

Spore suspensions were centrifuged at 10 000 × *g* for 5 min at 4 °C. Nucleic acid and protein release were assessed by measuring the absorbance of the supernatant at 260 and 280 nm (Evolution 60s, Thermo Scientific Inc., MA, USA).

For DPA leakage analysis, the supernatant was mixed 1:1 with a 50 μmol/L terbium (III) chloride solution (pH 5.6, Aladdin Industrial Co., Shanghai, China) and measured using a fluorescent microplate reader (Spark 10M, TECAN Industrial Co., Switzerland) with excitation at 270 nm and emission at 545 nm, following the method of Rao et al.[25]. Untreated spores and autoclaved spores (121 °C, 20 min) served as negative and positive controls, respectively. The DPA release (%) was calculated using the following equation (1):

$$\text{DPA release (\%)} = \frac{X_2 - X_1}{X_3 - X_1} \times 100 \quad (1)$$

Where X_1, X_2, and X_3 were the fluorescence intensity of untreated spores, OH or OB treated spores and autoclaved spores, respectively.

2.5 Determination of molecular structures by Fourier transform infrared spectroscopy (FTIR)

To analyze the molecular st ructures, spore pellets (untreated, OB, OH-500Hz, and OH-50Hz treated spores) were collected by centrifugation (10 000 × *g*, 5 min, 4 °C) and freeze-dried. FTIR analysis was performed using the potassium bromide tableting method[26]. A 2 mg sample was ground with 200–400 mg of dry pure KBr (AR grade) and analyzed using a Nicolet iS5 FTIR spectrometer (Thermo-Fisher, MA, USA) at 400–4 000 cm^{-1} with a resolution of 0.4 cm^{-1}.

2.6 Determination of permeability of spore inner membrane and cortex

The spore suspensions (autoclaved, untreated, OB, OH-500Hz, and OH-50Hz treated samples) were stained with 0.5 μmol/L SYTO 16 and 15 μmol/L propidium iodide (PI) (Invitrogen, Carlsbad, CA, USA). The mixtures were incubated in the dark for 15 min and then washed twice with PBS to remove excess dye. The suspension was analyzed by flow cytometry (Accuri C6, BD, USA). The data from these populations were fitted within the appropriate rectangles.

2.7 Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) observation of spores

Spore morphology was examined using SEM and TEM based on a previously reported method with some modifications[14]. Spore pellets (untreated, OB, OH-500Hz, and OH-50Hz treated samples) were obtained by centrifugation (8 000 × *g*, 15 min, 4 °C), fixed in 2.5% glutaraldehyde for 4 h, washed 3 times with PBS, and dehydrated in an ethanol series (30%, 50%, 70%, 80%, 90%, and 100%) for 15 min.

For SEM, cells were sprayed onto copper mesh, dried at the critical point (LEICA EM CPD, Wetzlar, Germany), coated with gold (EIKO IB-3, Tokyo, Japan), and then observed by SEM (Hitachi S-3400N, Tokyo, Japan) at 15 kV.

For TEM, cells were soaked in an acetone and epoxy resin solution for 24 h and polymerized for 48 h at 60 °C. The samples were subsequently sliced into 50 nm sections using the Leica EM UC7 (Leica, Wetzlar, Germany) and placed on copper mesh for observation by TEM (Hitachi HT7800, Tokyo, Japan) at 80 kV.

2.8 Analysis of quality parameters of liquid food

2.8.1 Detection of pH and viscosity

Milk, orange juice, soymilk, and broth (20 mL) were taken to measure the pH value using a pH meter (FE-20, Mettler Toledo, Zurich, Switzerland).

Milk, orange juice, and soymilk (1.8 mL) was taken to assess the viscosity using a rotary rheometer (DHR-2, TA Co., Ltd., Delaware, USA) with a 40.0 mm stainless steel parallel plate at 25 °C. The shear rate was increased to 100 s^{-1}.

2.8.2 Detection and calculation of color

The color of milk, orange juice, soymilk, and broth were assessed using a colorimeter (CM-3700A, Konica Minolta Co., Ltd., Tokyo, Japan). The total color difference (ΔE) was calculated by equation (2):

$$\Delta E = \sqrt{(L_1^* - L_0^*)^2 + (a_1^* - a_0^*)^2 + (b_1^* - b_0^*)^2} \quad (2)$$

Where ΔE was the total color difference with fresh samples; L_0^*, a_0^*, and b_0^* were the color values of fresh samples; L_1^*, a_1^*, and b_1^* were the color values of inoculated or treated samples. When ΔE was the total color difference with untreated inoculated samples, L_0^*, a_0^*, and b_0^* were the color values of untreated inoculated samples, L_1^*, a_1^*, and b_1^* were the color values of treated samples.

2.9 Statistical analysis

All treatments were conducted in triplicate. The data were analyzed using analysis of variance (ANOVA) followed by Duncan's multiple range test. These statistical analyses were performed using IBM SPSS Statistics (version 25.0, IBM Co., NY, USA). Figures were created using Origin Pro (version 2021, Origin Lab Co., Massachusetts, USA). The results are presented as mean values ± standard deviation (SD).

3. Results and discussion

3.1 Inactivation effects and changes in heat resistance of spores after OH and OB treatments

In the study, 2 temperatures (80 and 98 °C) were selected to investigate the structural changes of spores. At 80 °C, only a small number of spores are inactivated, while the vast majority are inactivated at 98 °C. Additionally, 98 °C represents the relatively stable maximum temperature achievable by the OH equipment. The inactivation effects of OH and OB treatments on spores at these temperatures are presented in Table 1. The results indicated no significant differences in reduction among OB, OH-500Hz and OH-50Hz at both 80 and 98 °C, allowing us to focus on analyzing the structural changes of the spores under these consistent inactivation conditions.

To evaluate the wet heat resistance of OH- and OB-treated spores, inactivation and D-values were calculated following wet heat treatment. An increase in reduction and a decrease in D-value indicate a reduction in wet heat resistance. As shown in Fig. 1 and Table 2, the heat resistance of OB treated spores increased with rising treatment temperature, consistent with the findings by Rao et al.[24], who observed an increase in wet heat resistance after treatment at 75 °C, attributing this to an enhanced activation effect that increased the number of spore colonies rather than an intrinsic increase in the heat resistance of individual spores. In contrast, the heat resistance of OH-500Hz and OH-50Hz treated spores initially decreased when at temperature of 60 and 70 °C, followed by an increase at higher temperatures. The wet heat resistance of spores is inversely correlated with the water content in spore core[27]. The observed decrease in heat resistance may be due to spore germination or increased permeability of the spore's inner

Table 1
Inactivation effects and DPA release of spores treated with OH and OB heating.

Parameters	Temperature (°C)	OB	OH-500Hz	OH-50Hz
Reduction (lg (CFU/mL))	80	1.19 ± 0.04^a	1.12 ± 0.07^a	1.12 ± 0.03^a
	98	5.56 ± 0.07^a	5.39 ± 0.22^a	5.65 ± 0.19^a
DPA release (%)	80	20.82 ± 1.06^a	10.76 ± 0.73^b	12.25 ± 0.84^b
	98	85.31 ± 1.07^a	83.19 ± 0.88^a	66.46 ± 0.88^b

Note: Different lowercase letters meant significant differences among different treatment groups ($P < 0.05$).

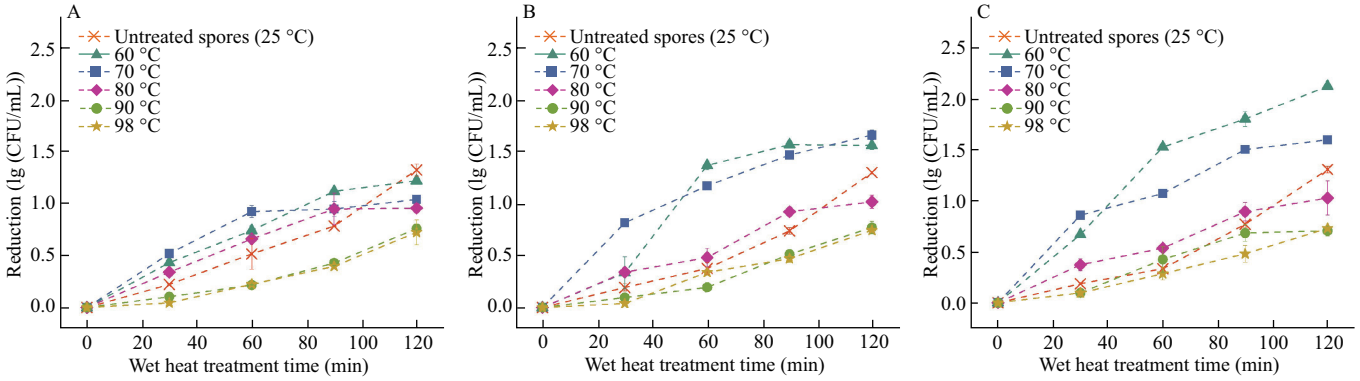

Fig. 1 Inactivation of (A) OB, (B) OH-500Hz, and (C) OH-50Hz treated spores by wet heat treatment at 80 °C. The initial population of spores were untreated spores (initial temperature of 25 °C) and spores treated by OB, OH-500Hz, and OH-50Hz to 60, 70, 80, 90, 98 °C, and then treated by wet heat treatment at 80 °C for 0–120 min.

Table 2
Decimal reduction times for wet heat treatment at 80 °C of untreated, OH and OB heating treated *B. cereus* spores.

OB or OH treatment temperature (°C)	D-values (min)		
	OB	OH-500Hz	OH-50Hz
25 (untreated spores)	93.53 ± 2.62^{Ac}	95.70 ± 0.46^{Ac}	94.35 ± 0.89^{Ac}
60	95.71 ± 1.37^{Ac}	69.13 ± 3.34^{Be}	55.87 ± 0.62^{Cc}
70	120.28 ± 7.92^{Ab}	75.47 ± 0.28^{Bd}	78.46 ± 1.54^{Bd}
80	125.83 ± 2.37^{Ab}	114.38 ± 3.27^{Bb}	119.51 ± 17.47^{ABab}
90	162.87 ± 6.62^{Aa}	154.76 ± 11.90^{Aa}	152.78 ± 13.89^{Aa}
98	169.68 ± 22.62^{Aa}	156.40 ± 4.89^{Aa}	163.48 ± 11.96^{Aa}

Note: Different lowercase letters meant significant differences among different treatment temperatures ($P < 0.05$). Different uppercase letters meant significant differences among different treatment groups ($P < 0.05$).

membrane[24]. This suggests that low-temperature (60–70 °C) OH-500Hz and OH-50Hz treatments may reduce spore wet heat resistance, potentially by increasing inner membrane permeability or promoting spore germination through non-thermal effects. The increased heat resistance observed at higher temperature (> 70 °C) may be attributed to the dominant role of thermal effects in spore inactivation.

3.2 Leakage of DPA, nucleic acids, and proteins

The inactivation of spores, accompanied by the release of intracellular substances, indicates damage to the cell's integrity. The wet heat resistance of spores can be enhanced by mineralization induced by metal ions and DPA[28]. As shown in Table 1, the release of DPA from OH-500Hz and OH-50Hz treatments at 80 °C was significantly lower (10.76% and 12.25%, respectively) compared to the OB treatment. However, at 98 °C, DPA release from the OB and OH-500Hz treatments increased to 85.31% and 83.19%, respectively, which were significantly higher than that of the OH-50Hz treatment (66.46%). Pillet et al.[29] found that pulsed electric field treatment caused partial destruction of the coat proteins and internal alterations of the cortex and core, but spore stiffness or volume was not altered. This result led to the assumption that neither Ca^{2+}-DPA release nor permeabilization of the inner membrane occurred. These results indicated that OB treatment caused the most severe membrane damage, while some spores treated with OH-50Hz retained DPA, and subsequently died due to other factors.

The release of nucleic acids and proteins from spores can be indicated by the absorbance of the supernatant at 260 and 280 nm. The absorbance values of the OB, OH-500Hz and OH-50Hz treatments increased significantly with rising temperature ($P < 0.05$), confirming membrane damage during processing. As shown in Fig. 2A, untreated spores (initial temperature of 25 °C) exhibited extremely low absorbance values at 260 and 280 nm, indicating that they effectively preserved the nucleic acids and proteins within the spore. There was no significant difference in absorbance values at 260 nm between the OB and OH treatments at 60 °C ($P > 0.05$). However, when the temperature reached 70 °C, the absorbance value of OH-500Hz treatment was significantly higher than that of the OB and OH-50Hz treatment groups. When the temperature reached 80 °C, the absorbance value of the OB treatment (0.121) was significantly higher than that of the OH-500Hz (0.060) and OH-50Hz (0.057) treatments. At 90 and 98 °C, the supernatant of the spores heated by OB and OH-500Hz had higher absorbance values than those of the OH-50Hz treatment, indicating more severe damage to the outer walls and membranes of the spores. OH-50Hz treatment consistently exhibited the lowest leakage of nucleic acids throughout the entire process.

Heat treatment can cause reversible breakage of sulfide and hydrogen bonds in certain enzyme proteins, leading to the release of proteins from the spores[30]. Among the treatments, the OB group had the highest absorbance values (0.029–0.181), whereas the OH-50Hz consistently maintained the lowest absorbance values (0.023–0.100) at 280 nm throughout the entire process (60–98 °C). The lowest leakage of nucleic acids and proteins from spores treated by OH-50Hz can be attributed to 2 possible explanations. Firstly, the trend in DPA leakage suggests that OH-50Hz caused less severe damage to the spore structure. Secondly, conformational changes in nucleic acids and solidify in protein caused by thermal and electrical effects may result in minimal leakage from the tiny pores in the cell membrane[31].

3.3 FTIR analysis of molecular structure of spore after OB and OH treatments

FTIR spectroscopy was used to further explore the changes in the cell membrane, peptidoglycan layer, nucleic acid, and proteins of *B. cereus* spores after treatments. The spectra changes resulting from different treatments are illustrated in Fig. 3A. The broad peak between 3 700 and 3 100 cm^{-1} was assigned to the N–H stretching of amide and O–H stretching of triterpene, polysaccharides, and sterols[32]. Compared to OB treatment, OH-treated spores showed significant changes in the 3 700–3 100 cm^{-1} range.

Principal component analysis (PCA) was utilized to visualize and distinguish the structural changes in the spores induced by OB and OH treatments. As shown in Fig. 3B, PC1 and PC2 explained 94.8% and 3.6% of the observed variances, respectively. This result indicated that the different treatments could be successfully separated by PCA, with PC1 playing a significant role in discrimination[33]. The distances among the 4 groups on the PCA plot reflect the varying degrees of change induced by different treatments. All treated spore groups were far away from the untreated spores (CT). At similar lethality, OH-50Hz treated spores were the most distant from the untreated spores, indicating that OH-50Hz exhibits the most distinct structural alterations compared to the untreated spores.

The 2nd derivative spectra figures were generated to reveal subtle distinctions in the original spectra (Figs. 3C and D). The wavelength range of 3 000–2 800 cm^{-1} primarily indicated alterations in membrane fatty acids and lipids within the spores. An increase in intensity within this spectral region range generally indicates conformational disorder within the cell membrane[34]. Specifically, the peak at 2 963 and 2 873 cm^{-1} primarily correspond to the C–H asymmetric and symmetric stretching bands of CH_3, while those at 2 930 and 2 852 cm^{-1} correspond to the asymmetric and symmetric stretching of CH_2[35-36]. After OB and OH treatments,

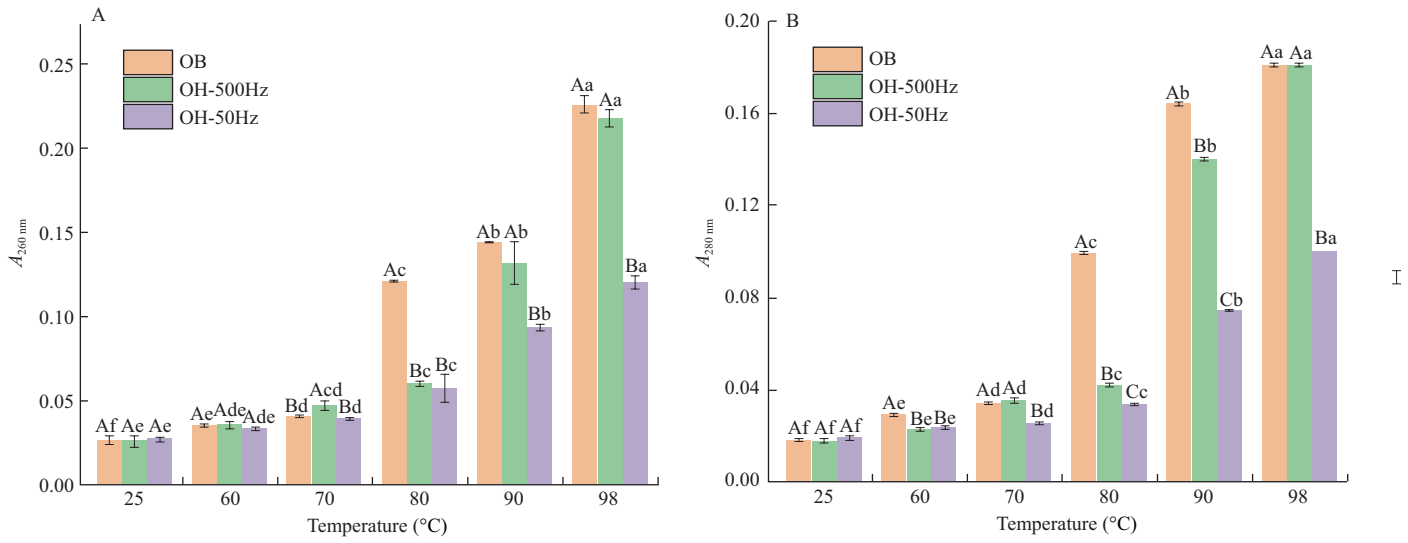

Fig. 2 Leakage of (A) nuclei acid-related, and (B) protein-related substances from spores during OB and OH treatments. Different lowercase letters means significant differences of the same treatment group under different processing temperatures ($P < 0.05$); different uppercase letters means significant differences among different treatment groups ($P < 0.05$).

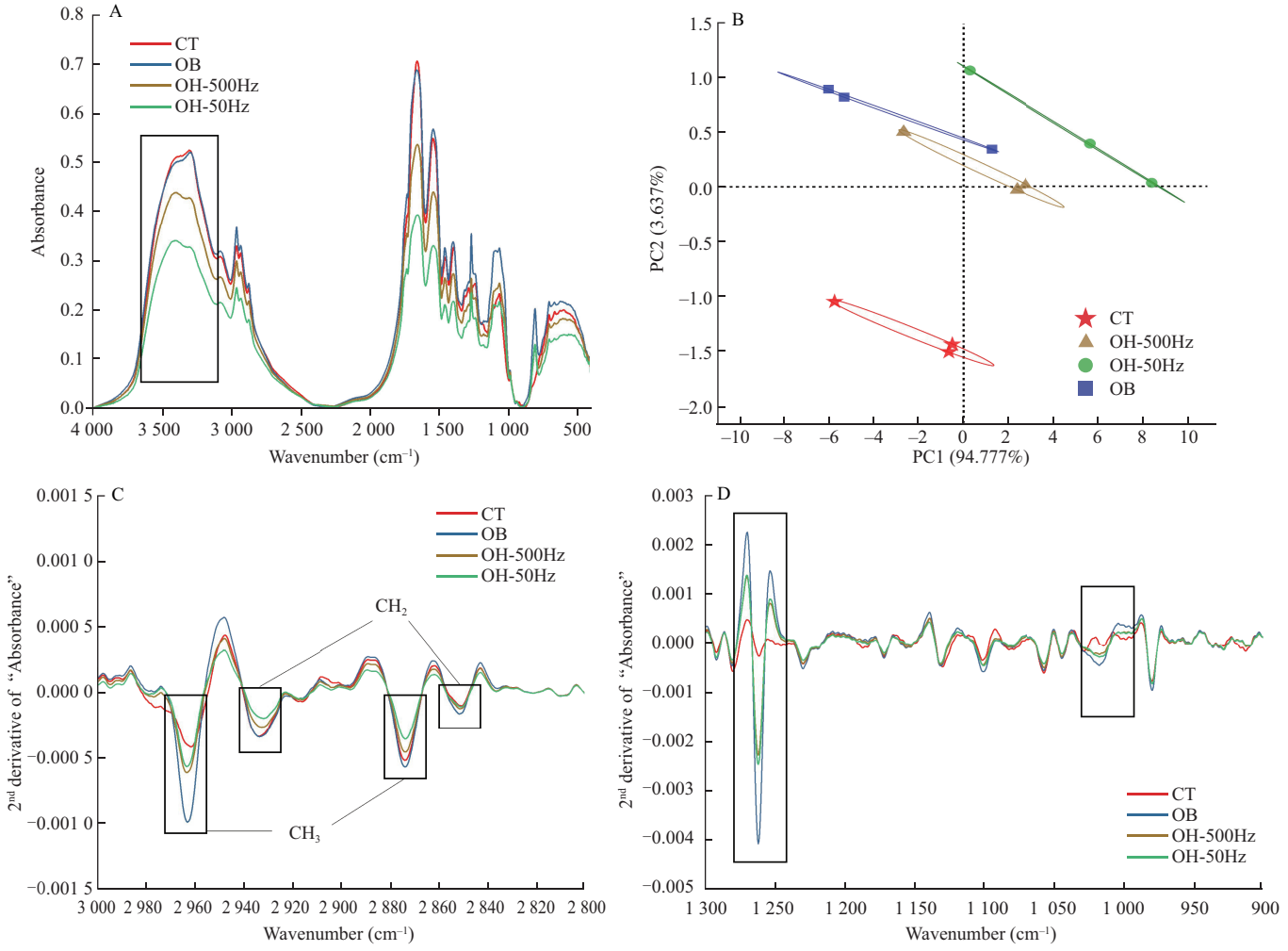

Fig. 3 FTIR analysis of molecular structure in spore treated by OB, OH-500Hz, and OH-50Hz to 98 °C. (A) FTIR spectra; (B) PCA analysis of FTIR spectra; (C) 2nd derived spectra of lipid region (3 000–2 800 cm^{-1}); (D) 2nd derived spectra of nucleic acid region (1 300–900 cm^{-1}).

the peaks around 2 963 cm^{-1} exhibited increased intensity and shifted positions compared to the untreated spores, indicating changes in the C–H stretching vibrations of fatty acids and lipids, which may affect the morphological structure of the inner membrane[34,37]. Compared to untreated spores, OB treatment resulted in a more significant increase in intensity at 2 852 cm^{-1}, while OH-50Hz showed the least intensity at around 2 932 cm^{-1}, indicating changes in lipid fluidity[38].

Furthermore, the spectral range of 1 300–900 cm^{-1} provided insights into the vibrational features of cellular proteins, nucleic acids, membrane constituents, and cell wall components[39]. The intensity of the peak at 1 261 cm^{-1} significantly increased after OB and OH treatments, signifying changes in the unordered structures of amide III[37]. Meanwhile, the region of 1 250–1 300 cm^{-1} exhibited 3 significant peaks that changed after OB and OH treatments (1 269, 1 261, 1 253 cm^{-1}). These changes may be associated with the vibrations of C–O from esters or carboxylic acids[40]. Additionally, the change in the absorption band at 1 030–980 cm^{-1} is mainly caused by the C–O–C stretching vibration, reflecting structural changes in the peptidoglycan layer of the cell wall after the treatments[41-42]. Therefore, OB and OH treatments affect fatty acids, lipids, esters, carboxylic acids, and the peptidoglycan layer, thereby altering the structure of the inner membrane and cortex, which play key roles in the resistance of spores[7-8].

3.4 Changes of spore cortex and inner membrane after OB and OH treatments

To assess the physiological heterogeneity in spore cortex and inner membrane damage in *B. cereus* spores, SYTO 16 and PI were used to stain DNA in spores with damaged or hydrolyzed cortex and inner membrane, respectively[24]. Dual-parameter dot plots were employed to visualize different subpopulations with distinct staining characteristics. Cells displaying in the Q1 area denoted as PI positive and SYTO 16 negative, cells in the Q2 area were denoted as PI positive and SYTO16 positive, cells in the Q3 area were denoted as PI negative and SYTO 16 negative, and cells in the Q4 area were denoted as PI negative and SYTO 16 positive. The scatter plots showed that untreated spores were predominantly found in the Q3 area (84.8%), indicating they were both PI negative and SYTO 16 negative, suggesting that both the cortex and inner membrane were intact (Fig. 4A), as they were PI negative and SYTO 16 negative. In contrast, autoclaved spores (Fig. 4B) were mainly found in the Q2 area (81.5%), indicating that both the cortex and inner membrane were damaged, as they were PI positive and SYTO 16 positive.

As the temperature increased, the percentage of spores staining positive for both PI and SYTO 16 (Q2) also increased. The PI (Q1 + Q2) and SYTO 16 (Q2 + Q4) staining percentages of the spores increased with the rising temperature. When the temperature reached 80 °C, the PI staining percentage of OH-500Hz and OH-50Hz treatments (Figs. 4E and G) was lower than that of OB treatment (Fig. 4C). At 98 °C, OH-50Hz treatment had the lowest PI staining percentage (Fig. 4H), while OB treatment had the highest (Fig. 4D). However, it is worth noting that the actual inactivation efficiency and DPA release percentage were significantly higher than the PI staining percentage, indicating that the inactivation of spores by OH does not solely rely on the inner membrane disruption. Some inactivated spores were not stained with PI, suggesting that while PI could act as an indicator of spore inactivation, it may not accurately represent the percentage of inactivated *B. cereus* spores[43]. Interestingly, OH-50Hz treated spores had the lowest SYTO 16 staining at 80 °C and the highest SYTO 16 staining at 98 °C, indicating that OH-50Hz treatment caused changes in the cortex of spores, leading to an increased SYTO 16 staining percentage. This finding is consistent with the study by Wang et al.[44], which reported that the inactivation of spores by moderate electric fields at mild temperatures (55–75 °C) is directly correlated with damage to the cortex and inner membrane.

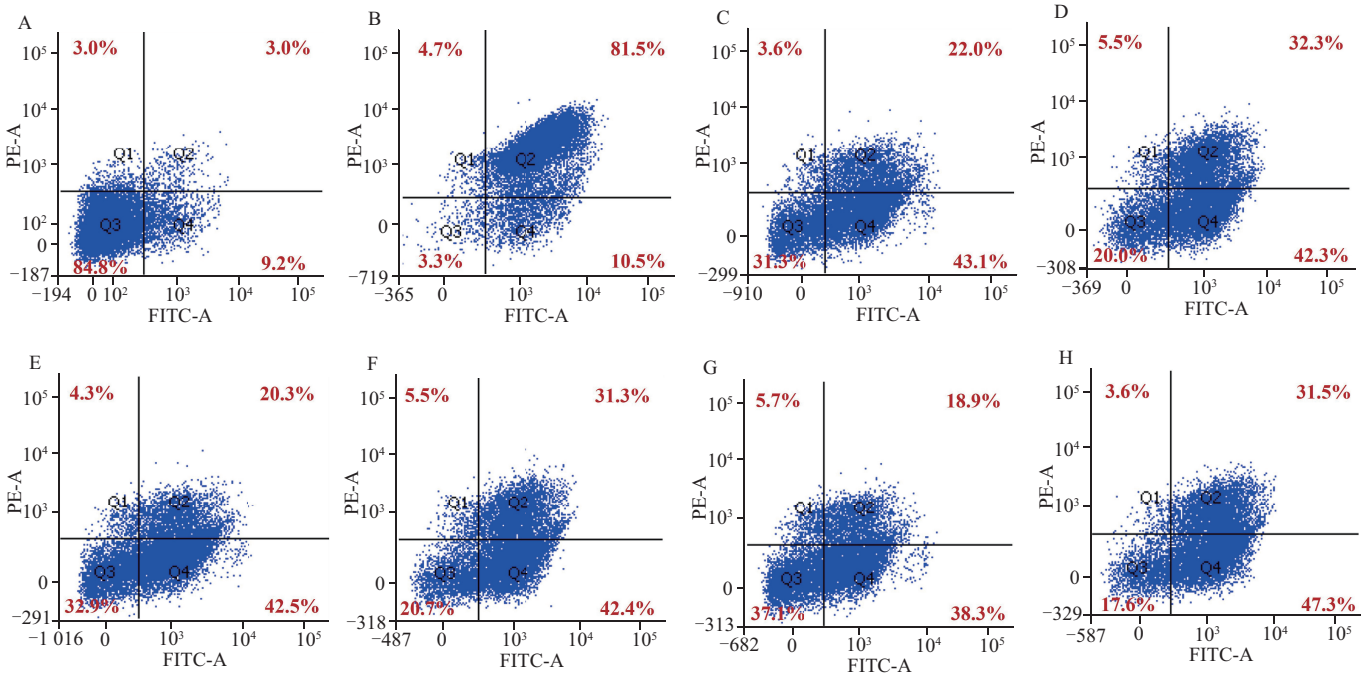

Fig. 4 Flow cytometry images of (A) untreated spores, (B) autoclaved spores; spores treated by OB to (C) 80 °C and (D) 98 °C; spores treated by OH at 500 Hz to (E) 80 °C and (F) 98 °C; spores treated by OH at 50 Hz to (G) 80 °C and (H) 98 °C.

3.5 Changes of spore structure after OB and OH treatments

The morphological changes of spores after OB and OH treatments were examined using SEM. As shown in Fig. 5A, the untreated spores were initially intact and cylindrical in shape, with irregular shrinkage and wrinkled ridges. As the processing temperature increased, the damage to the spore structure became more severe, leading to shrinkage and fractures, with a greater number of fragments forming at higher temperatures. Although both OB and OH treatments achieved similar inactivation effects, OB treatment resulted in the most severe damage, with a notable increase in the number of fragments. These observations are consistent with previous research, which reported that OH-treated endospores exhibited shrinkage and distorted outer spore coats[16].

The intracellular structure of untreated and OB- and OH-treated spores was assessed using TEM. As shown in Fig. 6A, untreated spores exhibited an intact exosporium, coat, cortex, and core. At 80 °C, some spores still retained good integrity, while others displayed signs of collapsing and severe deformation. OB, OH-500Hz and OH-50Hz treatments induced similar changes in spore structure at 80 °C (Figs. 6B, D and F). As the temperature reached 98 °C, more spores exhibited deformation, and the boundaries of internal structures became blurry, with the spore cores lightening in color, indicating severe damage to the multilayer structure and leakage of core contents. However, OB-treated spores showed distinctive characteristics, including a lack of observable cortex and dispersed core contents, along with decomposing cortex layers (Fig. 6C). OH-500Hz treated spores exhibited deformation and the loss of intracellular material (Fig. 6E), while OH-50Hz treated spores displayed unclear boundaries between individual layers, with the inner spore structure appearing white (Fig. 6G). These results suggest that the 3 treatments had distinct effects on the intracellular structure of the spores.

3.6 Potential spore-inactivation mechanism analysis

Similar inactivation effects were observed among the OB, OH-500Hz and OH-50Hz treatments when the temperature reached 80 and 98 °C (Table 1). However, the degree of damage to the spore structure and the leakage of intracellular substances varied among these treatments, with OH-50Hz exhibiting the least damage (Figs. 2, 4-6). The damage to spore structure and leakage of intracellular substances may contribute to spore death. Wet heat treatment can cause permeabilization of the inner membrane in spores[45]. Most spores inactivated by OB and OH-500Hz released DPA, whereas only 66.46% of spores inactivated by OH-50Hz released DPA (Table 1). The all-or-none phenomenon observed during DPA release suggests that no spore exhibited partial DPA release[46]. The primary inactivation mechanism of OH-50Hz may extend beyond changes in inner membrane permeabilization, as some OH-50Hz inactivated

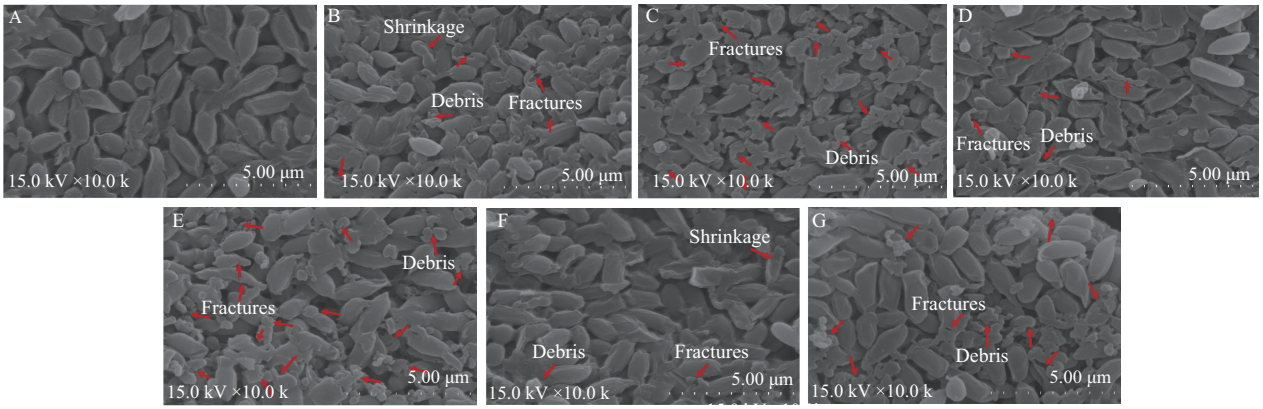

Fig. 5 SEM images of (A) untreated spores; spores treated by OB to (B) 80 °C and (C) 98 °C; spores treated by OH at 500 Hz to (D) 80 °C and (E) 98 °C; spores treated by OH at 50 Hz to (F) 80 °C and (G) 98 °C.

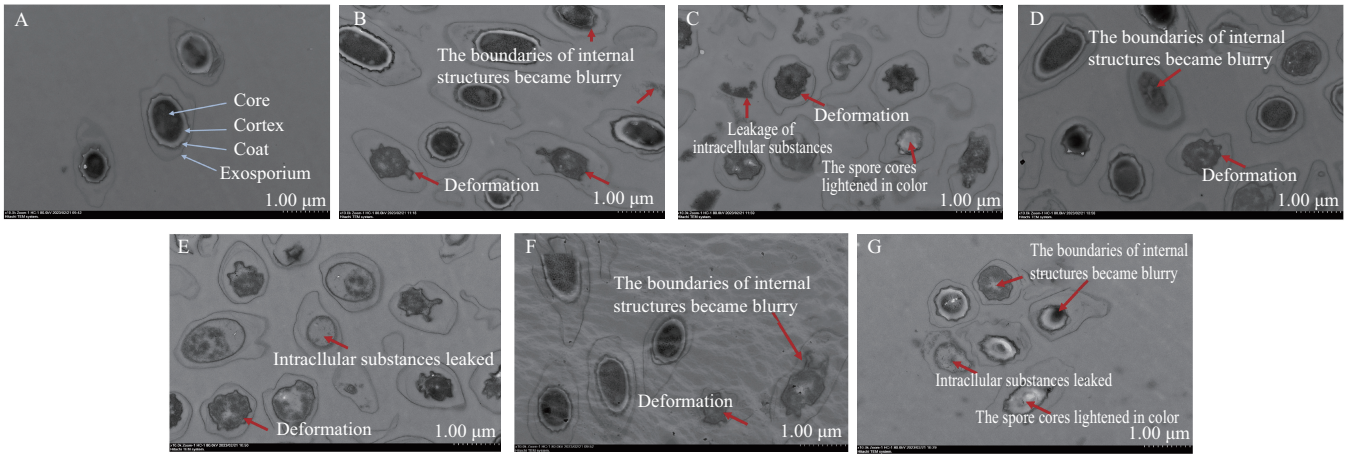

Fig. 6 TEM images of (A) untreated spores; spores treated by OB to (B) 80 °C and (C) 98 °C; spores treated by OH at 500 Hz to (D) 80 °C and (E) 98 °C; spores treated by OH at 50 Hz to (F) 80 °C and (G) 98 °C.

spores (33.54%) retained DPA. This indicates that OH-50Hz may target additional components, such as germination receptor proteins in the inner membrane or the spore core, beyond inner membrane permeabilization. These findings align with the work of Schottroff et al.[15], which suggested that electric fields may target the SASPs-DNA complex in the spore core.

Furthermore, significant differences in heat resistance were observed between the OB and OH treatments. The OH treatments decreased the heat resistance of spores at lower temperatures (i.e., 60–70 °C), potentially promoting subsequent inactivation. In contrast, OB treatment increased spore heat resistance throughout the entire process. However, OH did not further reduce the permeability of the spore inner membrane compared to OB, as indicated by the results of the intracellular substances leakage. Therefore, low temperature (60–70 °C) OH-500Hz and OH-50Hz treatments reduced the heat resistance of spores by inducing spore germination.

Heat activation of spores at low temperature can render them more susceptible to germinant, alter their germination characteristics, and increase their germination rate[47-48]. The heat-activated spores in this case, retained heat resistance and refractive properties[49]. However, as the treatment temperature increased, the process transitioned from "heat activation" to "heat injury", leading to thermal damage accumulation and increased germination heterogeneity[50-51]. The germination process of *Bacillus* spores involves several stages, including stage 1 (CaDPA release, partial core hydration, and some resistance loss) and stage 2 (Cortex hydrolysis, full core hydration, core expansion, and significant loss of resistance)[52]. However, the percentage of cortex damage (38.3%–43.1%) was higher than the percentage of DPA release (10.76%–20.82%) in this study (Table 1, Figs. 4 and 6), indicating that most of the cortex damage occurred prior to DPA release, which is not in accordance with the germination process, thus most of the OH-treated spores did not germinate[53]. Consequently, only a small proportion of spores germinated, reflecting germination heterogeneity. Lv et al.[22] also reported that thermal treatment at 80 °C could compromise the cortex integrity but had little effect on the DPA release. The possible inactivation mechanism of *B. cereus* spores by OH is proposed in Fig. 7, which has been preliminarily discussed and clarified in this study.

3.7 *Effects of OH and OB on the quality of milk, orange juice, soy milk and broth*

The impact of OH and OB treatments on various quality parameters of different liquid food products, including milk, orange juice, soy milk, and broth, was investigated. The results are presented in Table 3.

As shown in Table 3, both OH and OB treatments effectively inactivated *B. cereus* spores in all liquid food products, achieving a final reduction of 5.68–5.99 (lg (CFU/mL)), with no significant differences observed between OH and OB ($P > 0.05$). Notably, OH treatment required a shorter duration than OB to achieve the same level of inactivation at a final temperature of 98 °C for milk, juice, and broth. Conversely, OH treatment necessitated more time for soy milk due to its lower electrical conductivity (Table S1).

The pH values of liquid food products were not significantly affected by inoculation with *B. cereus* spores. Additionally, the pH values of milk, soy milk, and broth remained relatively stable following both OH- and OB-treatment. However, the pH value of orange juice increased after both treatments, possibly due to the diffusion of materials or salts from the spores into the juice[54].

Inoculation with *B. cereus* spores resulted in an increase in the viscosity of milk, likely attributed to the production of heat-resistant proteases by the spores[55]. In contrast, no significant changes in viscosity were observed for juice and soy milk after spore inoculation. Interestingly, the viscosity of both OH- and OB-treated milk was lower than that of untreated milk, potentially due to changes in fat globule size and alterations in the tertiary structure of milk proteins[56]. Conversely, the viscosity of orange juice decreased after following both treatments, while the viscosity of soy milk increased, likely due to water loss and an increase in solid content during thermal treatment.

The total color differences between fresh samples and untreated inoculated samples of these liquid foods after OH and OB treatments were also analyzed. The total color differences in orange juice and broth were greater than those in milk and soy milk after inoculation with *B. cereus* spores, attributable to the natural coloration of the materials. The *a** value, representing the red-green attribute, was significantly higher in OB-treated juice compared to OH-treated juice, indicating a redder coloration. In contrast, OH-treated soy

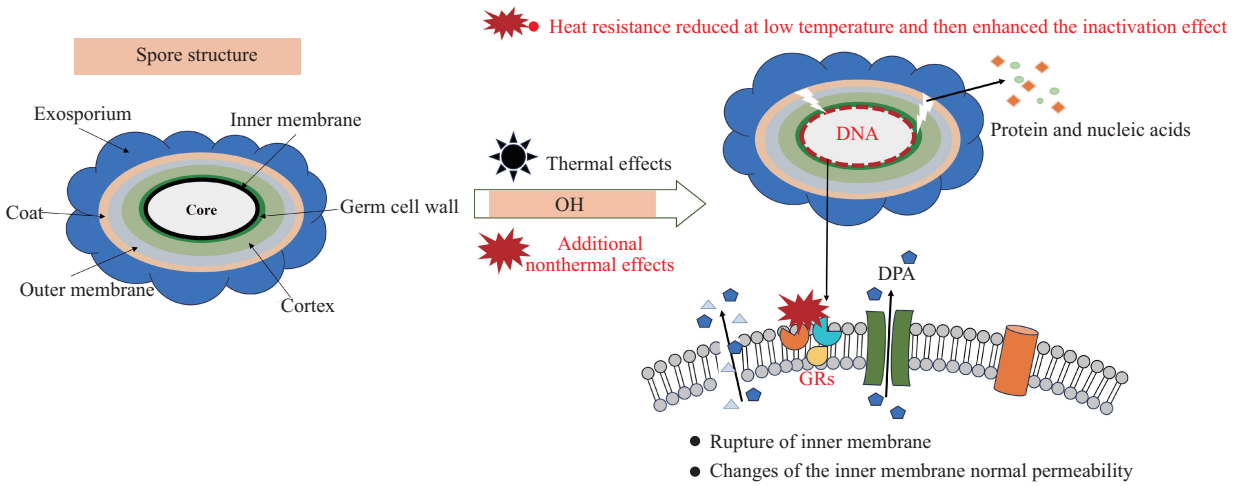

Fig. 7 Schematic diagram of *B. cereus* spore inactivation mechanism by low frequency OH.

Table 3
Inactivation efficiency and quality changes of milk, orange juice, soy milk, and broth treated with OH and OB.

Samples	Treatments	Reduction in spore counts (lg (CFU/mL))	pH	Viscosity at 100 s^{-1} in mPa·s	L^*	a^*	b^*	ΔE_1	ΔE_2
Milk	Fresh	–	6.72 ± 0.01^{a}	1.36 ± 0.09^{b}	93.03 ± 0.01^{a}	-1.88 ± 0.00^{c}	7.09 ± 0.01^{d}	–	–
	Inoculated	–	6.73 ± 0.02^{a}	1.79 ± 0.10^{a}	92.77 ± 0.05^{b}	-1.62 ± 0.01^{a}	7.28 ± 0.01^{a}	0.40 ± 0.03^{a}	–
	OB	5.99 ± 0.05^{a}	6.71 ± 0.03^{a}	0.79 ± 0.04^{d}	92.71 ± 0.01^{c}	-1.82 ± 0.01^{b}	7.18 ± 0.01^{c}	0.34 ± 0.01^{b}	0.22 ± 0.00
	OH	5.93 ± 0.09^{a}	6.73 ± 0.01^{a}	0.98 ± 0.04^{c}	92.63 ± 0.06^{d}	-1.81 ± 0.01^{b}	7.21 ± 0.01^{b}	0.42 ± 0.05^{a}	0.25 ± 0.02
Orange juice	Fresh	–	3.85 ± 0.01^{b}	1.15 ± 0.02^{a}	55.90 ± 0.06^{b}	0.55 ± 0.01^{c}	30.69 ± 0.02^{b}	–	–
	Inoculated	–	3.84 ± 0.00^{b}	1.16 ± 0.01^{a}	56.99 ± 0.07^{a}	0.95 ± 0.01^{b}	31.58 ± 0.02^{a}	1.47 ± 0.05^{b}	–
	OB	5.71 ± 0.20^{a}	3.90 ± 0.01^{a}	0.78 ± 0.00^{b}	53.94 ± 0.04^{c}	1.28 ± 0.01^{a}	28.83 ± 0.02^{c}	2.79 ± 0.04^{a}	4.12 ± 0.04
	OH	5.75 ± 0.21^{a}	3.88 ± 0.01^{a}	0.59 ± 0.05^{c}	53.85 ± 0.06^{d}	0.96 ± 0.01^{b}	28.86 ± 0.05^{c}	2.77 ± 0.07^{a}	4.15 ± 0.07
Soy milk	Fresh	–	6.59 ± 0.01^{a}	0.91 ± 0.09^{bc}	83.32 ± 0.02^{a}	0.07 ± 0.01^{c}	13.49 ± 0.02^{c}	–	–
	Inoculated	–	6.59 ± 0.04^{a}	0.80 ± 0.07^{c}	83.36 ± 0.05^{a}	0.15 ± 0.01^{a}	13.43 ± 0.01^{d}	0.12 ± 0.02^{b}	–
	OB	5.79 ± 0.31^{a}	6.56 ± 0.03^{a}	1.02 ± 0.05^{b}	83.11 ± 0.10^{b}	0.02 ± 0.01^{d}	13.69 ± 0.02^{a}	0.30 ± 0.07^{a}	0.39 ± 0.06
	OH	5.68 ± 0.27^{a}	6.59 ± 0.01^{a}	1.35 ± 0.12^{a}	83.32 ± 0.03^{a}	0.11 ± 0.01^{b}	13.64 ± 0.02^{b}	0.16 ± 0.02^{b}	0.22 ± 0.01
Broth	Fresh	–	7.22 ± 0.01^{a}	–	92.12 ± 0.02^{a}	-0.88 ± 0.00^{d}	14.26 ± 0.01^{d}	–	–
	Inoculated	–	7.24 ± 0.04^{a}	–	79.03 ± 0.04^{d}	0.47 ± 0.00^{a}	18.01 ± 0.01^{a}	13.68 ± 1.82^{a}	–
	OB	5.99 ± 0.13^{a}	7.24 ± 0.02^{a}	–	83.42 ± 0.03^{c}	0.16 ± 0.00^{b}	17.94 ± 0.01^{b}	9.51 ± 0.03^{b}	4.40 ± 0.03
	OH	5.76 ± 0.20^{a}	7.22 ± 0.01^{a}	–	87.43 ± 0.25^{b}	-0.28 ± 0.02^{c}	17.61 ± 0.02^{c}	5.80 ± 0.21^{c}	8.44 ± 0.25

Note: OB and OH refer to samples treated with OB and OH with OH-50Hz , respectively. ΔE_1 and ΔE_2 refer to the total color difference compared to fresh samples and untreated inoculated samples, respectively. Different lowercase letters meant significant differences among different groups ($P < 0.05$).

milk and broth exhibited colors closer to their untreated samples. These differences in color changes may be related to various factors, including the inherent color of the food materials, spore inactivation, and chemical reactions such as the Maillard reaction[13].

4. Conclusion

In conclusion, OH treatment effectively inactivates *B. cereus* spores in various liquid food products without significantly compromising the quality of the liquid food. The application of OH treatments reduce the wet heat resistance of spores at a lower temperature, facilitating subsequent spore inactivation. Our comprehensive analysis of spore damage revealed that OH treatments result in damage to multiple spore components, including protein, nucleic acid, and DPA leakage, as well as structural damage to the spore coat, cortex, inner membrane and core. We propose a novel hypothesis that OH at 50 Hz may exert additional effects on the germinant receptor proteins or the spore core, which warrants further investigation. A deeper exploration of the molecular mechanisms by which OH impacts spore proteins and germination processes is essential. In summary, this research offers valuable insights into the effectiveness of OH treatment for spore inactivation and its potential application in liquid food systems. The findings indicate that OH treatment could be a promising method for enhancing food safety by eliminating *B. cereus* spores.

Conflict of interest

All authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2025.9250470.

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