

Methods of destroying bacterial spores

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The spores of *Bacillus* spp. are found widely distributed in nature, which may cause contamination of the environment and eventually diseases to humans and animals. The formation of the bacterial spore is a sophisticated mechanism by which some bacteria remain viable inadequate when subjected to survival stress such as lack of nutrients. The spores are highly resistant to heat, radiation, various disinfectant and antiseptic agents that routinely destroy bacteria in vegetative form. The inactivation of bacterial endospores has been regarded as a challenge for human health, environmental quality and food safety.

Keywords Spores; *Bacillus* spp.; Inactivation.

1. Introduction

When nutrients are limited, bacteria of the genus *Bacillus* form spores, metabolically inactive, which are extremely resistant to environmental changes and toxic chemicals that easily destroy their cellular counterparts [1-4].

With the resistance of the micro-organisms, spores, become also more resistant to antimicrobial treatments. According to Oliveira et al. [5], these micro-organisms have assumed great importance, especially in matters related to health conditions due to the waste discarded in the environment, such as fecal waste. Widely distributed in nature, the spores cause deterioration in food and eventually lead to human diseases.

Another important concern is the availability of drinking water, or at least useful, for public consumption. One possibility to solve this problem is the need to purify the water so that it can be reused by removing the existing waste therein. In this case, spores and other pathological micro-organisms constitute a serious hazard to public health. Although chlorine is effective in inactivating bacteria and viruses, the formation of potentially toxic and carcinogenic by-products is a matter which triggers the interest in alternative treatments [6].

Bacillus spp. are often present in raw milk and play an important role in the spoilage of milk and milk products [7]. The use of ultrahigh-temperature (UHT) processing should inactivate the spores of *Bacillus* species and result in fluid milk products with a long shelf-life without refrigeration. However, defects in UHT milk product stability have been reported [8,9]. This nonstability appeared to be caused by the presence of highly heat-resistant bacterial endospores which were first detected in UHT milk from southern Europe in 1985 and were later identified in other countries both in and outside Europe [8]. It thus became necessary to develop more efficient processes to inactivate this micro-organism completely and ensure milk commercial sterility.

As described in history, *Bacillus anthracis* spores were used as biological weapons. Thus, due to the high level of resistance, the most effective decontamination test is one that has the ability to destroy spore [10].

1.1. *Bacillus* spp. spores

The spores of *Bacillus* spp. are formed in a sporulation protection process which is usually induced by low levels of nutrients and under unfavorable survival conditions for the micro-organism in the environment. The formation of the bacterial spore is a sophisticated mechanism by which some bacteria remain viable and they produce a multilayer protective capsule fused to DNA [11]. In this way, spore forming bacteria are more resistant to antimicrobial treatments.

The formation of spores generates a type of cell which can survive for long periods with little or no nutrients, but is able to regain their vegetative form when environmental conditions become suitable for their survival. As a result, the spores have acquired a multitude of mechanisms to protect itself from damage caused by the different treatments. And this resistance, as well as the ubiquity of spores in the environment, makes these micro-organisms the main agents of food spoilage caused by these diseases and contaminated food [12]. Setlow et al. [13] reported that spores treated with strong alkali seem to be destroyed, but when they are grown in a rich medium it becomes feasible restarting the germination exogenous lysozyme. Thus, after destroying the spores is necessary to seed them in a rich medium to verify the viability of these organisms.

The spores are relatively resistant to many disinfectants and antiseptics that routinely destroy bacteria in vegetative form such as alcohols, phenols and chlorhexidine [11,14]. Agents commonly cited as anti-spores are represented by heat above 121°C by formaldehyde or glutaraldehyde and sodium hypochlorite solutions [15,16].

1.2. What makes the spores as resistant to various treatments?

The structure and chemical composition of spore play an important role in bacterial resistance. The spore has a structure very different from a cell in growth with a number of features and components of the original spores. Morphologically, from outside to inside, layers of the spore include: exosporo, coating layer, outer membrane, cortex, germ cell wall, inner membrane and core. The exosporo, an expanded version of the coating layer, is a structure found in spores of certain species, in particular *Bacillus cereus* [12, 17-20], and it composed of unique proteins, including glycoproteins [18,19,21,22]. Although the function of these proteins and exosporo are not fully understood, given the many pathogenic strains, such as *B. cereus* group, these structures may be important in the interaction of the spore with the target organism [12].

The spore coat layer is a complex structure consisting of multiple layers, most of which are products of specific genes spores [17,18].

The outer membrane cannot maintain the integrity of the spores inactive and therefore does not represent significant barrier permeability. According to Nicholson et al. [3] and Setlow [4], removal of outer membrane proteins together with the coating layer does not have any remarkable effect on the resistance of spores against heat, radiation and certain chemicals.

The cortex is composed of peptidoglycan (PG) having a structure similar to the vegetative form, but various changes in specificity [23]. It is essential for the formation of spores inactive and reduces the water content of the core thereof. Besides, the cortex protects the inner membrane of the spore, becoming a major barrier to the entry of small hydrophilic molecules in the central portion of the core spores. During germination of the spores, the cortex is degraded and this mechanism is essential for the expansion of the core of spores and subsequent growth [24].

The germ cell wall is also composed of peptidoglycan and probably plays no role in spore resistance, but makes up the cell wall of the spore external growth [12].

The inner membrane, in contrast to the outer membrane of the spores, is a strong permeability barrier and assigning plays an important role in spore resistance to many chemicals, particularly those which pass through the membrane causing damage to the DNA of spores [3,4,25]. It is very compact in dormant spores, duplicate volumes covering the first minutes of germination in the absence of energy production [26].

The final layer of the spore is the core analogous to the growth of cells in plasma and contains enzymes, as well as DNA, ribosomes and RNAs. In almost all cases, the spore enzymes and nucleic acids are identical to growing cells [12].

There are also two small core molecules which are important in the resistance of the spores. The first is the water, it comprises 75-80% of the protoplasm wet weight of a cell growing with comparison of spores, only 27-55% of the core, depending on the species. The low moisture content of the core is probably the main factor in enzyme inactivation and most important factor to determine the resistance of the spores to moist heat [25].

The second important molecule in the core is the pyridine-2,6-dicarboxylic acid (dipicolinic acid). This molecule comprises 5-15% of the dry weight of the spores of *Bacillus* spp. [12] and contributes to the inactivated state of the spores and dehydration core, which confers resistance to moist heat [4,27,28]. In addition, there are small acid soluble proteins that protect the DNA spore from damage caused by heat, ultraviolet radiation and some chemicals genotoxic such as nitrous acid [4,29].

2. Methods of destroying *Bacillus* spp. spores

Disinfections of micro-organisms in liquid are an important procedure in medicine [30] and the food industry. The method that has been adopted to inactivate the micro-organisms in a liquid is the heat pulse method [31], which applies heat to the liquid for a short period of several minutes. The temperature of the pressurized water increases to 130°C at 200 kPa, and micro-organisms including *Bacillus* spores are inactivated. However, any liquid to be sterilized tends to suffer modification in taste and aroma, in spite of the short period of heat application [32].

According Hayashi et al. [32], the atmospheric plasma torch produced by a barrier discharge using air and oxygen as working gases is adopted to inactivate micro-organisms in a liquid. In their studies, they investigated and compared the inactivation characteristics of *Bacillus* spore with the heat sterilization method, and sterilization factors are active species with short-lifetimes produced in water. The population of *Bacillus thuringiensis* spore in pure water decreases 10^{-3} times within 20 minutes, and the obtained result is equivalent to the heat sterilization of 95°C. All tendencies of the survival curves with the plasma treatments are similar to those with the heat treatment. This fact implies that the inactivation mechanism by the plasma irradiation would be same as the heat treatment that is modification and decomposition of proteins contained inside the cell wall.

Bacillus sporothermodurans is a milk spoilage bacterium producing resistant endospores that survive ultra-high temperature treatment. The combination of heat with high hydrostatic pressure (HP) has been proposed as an interesting approach for the inactivation of bacterial spores in food [33]. HP has been shown to be efficient at destroying vegetative bacteria and yeasts although bacterial spores appear to be more resistant to HP [34]. Aouadhi et al. [35] studied the inactivation of *B. sporothermodurans* LTIS27 spores by combined hydrostatic pressure and heat treatments. A central

composite experimental design was used to evaluate the effect of pressure (300-500 MPa), temperature (30-50°C), and pressure-holding time (10-30 min) on the inactivation of spores in distilled water and skim milk. The inactivation observed was shown to fit well with the values predicted by the quadratic equation, since R^2_{adj} were 0.970 and 0.977 in distilled water and milk, respectively. By analyzing the response surface plots, the inactivation was shown to be higher in distilled water than in milk under all the conditions tested. This was probably due to a protective effect of milk against inactivation by pressure. The optimum process parameter values for a 5-log cycle reduction of spores were calculated as 477 MPa/48°C for 26 min and 495 MPa/49°C for 30 min in water and in milk, respectively. This study shows the efficiency of hydrostatic pressure in combination with moderate temperature to inactivate *B. sporothermodurans* spores. Such treatments could be applied by the dairy industry to ensure the commercial sterility of UHT milk.

Research studies involving ultrasound processing for the food industry have been increasing since cavitation is a fundamental principle for destroying and removing micro-organisms from fresh food surfaces [36,37]. Ultrasound treatment can reduce levels of bacterial spores by inactivating their enzymes, though spores are generally known to be more resistant than vegetative cells. Regarding this effect, critical processing factors of ultrasound treatment are variable and involve multiple factors such as characteristics of the ultrasonic waves, treatment time, the type of pathogens and food. However, the effects are not exerted enough for inactivation of bacterial spore when using ultrasound alone [38]. Mostly, the bath type ultrasound in the range of 20-100 kHz has been utilized by industry because ultrasound at these frequencies can generate a more powerful cavitation phenomenon. However, ultrasound treatment has limitations for application by industry because cavitation phenomenon may be detrimental to prejudicial effects on food quality [39,40].

Bacillus cereus is an important foodborne pathogen causing diarrheal and emetic types of food poisoning. This organism has been often isolated from foods and implicated in many foodborne outbreaks involving raw and pasteurized milk and dairy products [41], skim milk powder [42], fresh vegetables and ready-to-eat vegetable-based foods [43-46]. Chemical sanitizers are commonly used to control foodborne pathogens on produce. For example, chlorine and hydrogen peroxide are easy to handle, very inexpensive, and are soluble in water and stable over a long storage time [47]. However, *B. cereus* spores are reportedly less permeable to antimicrobial agents than are the corresponding vegetative cells because spore DNA is able to protect from these agents of DNA damage [48,49]. Hydrophobic properties of *B. cereus* spores may potentially reduce sanitizer contact and penetration. Therefore, these bacteria are important in the produce industry since they can survive most chemical and heat processing.

Sagong et al. [50] compared the effectiveness of ultrasound treatment singly and in combination with surfactants as an alternative method to conventional sanitizers containing chlorine for reducing numbers of *B. cereus* spores on fresh produce. A cocktail of three strains of *B. cereus* spores was inoculated onto iceberg lettuce and then treated with ultrasound for 0, 5, 10, 20 and 60 min. Five minutes was found to be an adequate ultrasound (40 kHz, 30 W/L) treatment time which also caused no damage to lettuce leaf surfaces as observed through a field-emission scanning electron microscope. Iceberg lettuce and carrots were inoculated with a cocktail of three strains of *B. cereus* spores and treated with combinations of ultrasound and various concentrations (0.03 to 0.3%) of surfactant (Tween 20, 40, 60, 80 and Span 20, 80, 85) solutions for 5 min. The efficacy of the combination of ultrasound and surfactant increased depending on the hydrophile-lipophile balance (HLB). They observed the most effective treatment for reducing levels of *B. cereus* spores was the combination of ultrasound and 0.1% Tween 20, yielding reductions of 2.49 and 2.22 log CFU/g on lettuce and carrots, respectively, without causing deterioration of quality. These reductions were 1 log greater than those obtained by immersion in 200 ppm chlorine for 5 min.

Various chemical compounds are used as disinfectants in the food industry, in catering facilities, in restaurants, in hotels, and even in the home to ensure the microbiological safety of food. Nearly all of the disinfectants used have no sporicidal effect [51-55].

Chlorination has been a general method for decontamination of deadly bacteria either in the form of vegetative cells or in the form of spores since few decades. However, *B. anthracis* Sterne, *B. cereus* and *B. thuringiensis* exhibited noticeable resistance towards chlorination disinfection treatment [56]. Chemical disinfectants like calcium hypochlorite, free available chlorine, sodium hypochlorite, hydrogen peroxide [14], peracetic acid, formaldehyde, glutaraldehyde, sodium hydroxide, propylene oxide, cupric ascorbate and phenol and gases like ethylene oxide, chlorine dioxide, hydrogen peroxide plasma, methylene bromide and ozone showed promising sporicidal activities. Of these, hypochlorite, peracetic acid showed 99.9% inactivation efficiency against spores of *Bacillus subtilis* and this bacteria was reported as one of the surrogates of *B. anthracis* [14,57]. Heat treatment, UV, and gamma radiation also exhibited promising decontamination properties against spores of *Bacillus* species. These reports have also indicated that, *B. thuringiensis* and *B. anthracis* Sterne along with *B. cereus* form a better group of surrogates for virulent *B. anthracis* [16,58].

Although above decontaminants exhibited superior decontamination properties, certain disadvantages were also observed. The active chlorine content decreases with storage time, and bleach is corrosive to many surfaces. The chlorination treatment was observed to produce toxic by-products. Organic decontaminants leave large amount of non-biodegradable waste. Hydrogen peroxide and oxone based decontaminants lack stability. Inactivation of spores by photocatalytic oxidation method is one of the promising approaches, which eludes the production of toxic by-products

as observed in the case of chemical disinfectants. Moreover, application of sun light along with nano-titania is environmentally benign method for decontamination of biological warfare agents [59].

Prasad et al. [59] studied the kinetics of inactivation of spores of *B. anthracis* Sterne by using nano-sized titania and UVA radiation (in mW/cm² which is safe) or sun light. Synthesized as well as commercial samples have been used for the above experiments. They concluded the spores of *B. anthracis* Sterne have been efficiently inactivated by using titania nanomaterials and UVA light or sun light. The inactivation kinetics demonstrated pseudo first order behaviour. Spore death can be attributed to the leakage of cellular fluids due to damage of spore as observed by scanning electron microscopy data and DNA damage. Sun light also exhibited superior decontamination properties against *B. anthracis* Sterne spores.

Heat-resistant micro-organisms such as *B. subtilis* spores derived from soil may exist in soybean milk and can reduce the shelf life of tofu produced from conventional processing of soybean milk. Conventional high-temperature, long-time heat processing of soybean milk sufficiently inactivates spores of this organism but negatively impacts the gel strength of the resultant tofu due to protein denaturation [60]. The ohmic heating method is older than microwave heating and was reportedly used to inactivate micro-organisms in milk in 1919 [61].

Until recently, ohmic heating was commonly thought to kill micro-organisms through a thermal effect. However a growing body of evidence suggests that non-thermal effects may occur [62]. Today, ohmic heating using a frequency of around 20 kHz has become a useful technology in the food industry and has been used to process fish cake since 1990 because of the increased stability and increased energy efficiency [63].

Little literature exists regarding the non-thermal effect of electricity on bacterial spores during ohmic heating. In the absence of adequate understanding of spore inactivation kinetics, the method and conditions for ohmic sterilization are typically selected on the basis of existing purely thermal kinetic data [64,65]. New methods are needed for efficient bio-validation of ohmic heating in order to realize the full potential of the process. If additional non-thermal effects of electricity exist, it may be possible to reduce process severity, potentially improving quality [62].

Uemura et al. [63] studied radio-frequency heating to soybean milk through a Teflon film to process *B. subtilis* spores in milk and examined the effectiveness of inactivation. Tofu was then made from conventional heat-treated soybean milk, RF-FH heated soybean milk, and another conventional heat-treated soybean milk, and their gel strengths were compared. The following results were obtained when soybean milk was processed at a frequency of 28 MHz by RF-FH using non-contact heating through an insulating film. RF-FH processing reduced *B. subtilis* spores in soybean milk by four-logarithmic orders at an outlet temperature of 115°C. Teflon film covering the electrodes effectively prevented scaling when treating soybean milk. Tofu made from RF-FH-treated soybean milk had a higher breaking strength than tofu made from conventionally heated soybean milk. It was concluded that RF-FH could be used to improve soybean safety and tofu quality.

Somavat et al. [62] determined the kinetics of inactivation of *Geobacillus stearothermophilus* spores under ohmic and conventional heating using a specially constructed test chamber with capillary sized cells to eliminate potential sources of error and ensure that identical thermal histories were experienced both by conventionally and ohmically heated samples. Ohmic treatments at frequencies of 60 Hz and 10 kHz were compared with conventional heating at 121, 125 and 130°C for four different holding times. Both ohmic treatments showed a general trend of accelerated spore inactivation. It is hypothesized that vibration of polar dipicolinic acid molecules and spore proteins to electric fields at high temperature conditions may result in the accelerated inactivation.

Very few data have been reported in the literature to characterize a fog system's operation and fog environment, and how operational parameters may affect biocidal efficacy. Should fogging of sporicidal decontaminants prove to be efficacious in inactivating bacterial spores, it could potentially represent a relatively easy to use and inexpensive building decontamination technology (compared to fumigation techniques) that could be employed in the event of a wide area release of *B. anthracis* spores [66].

Wood et al. [66] evaluated the operation and effectiveness of a commercially available fogging device to inactivate bacterial spores in a pilot-scale decontamination chamber. The sporicidal decontaminant chosen for testing was a solution of hydrogen peroxide/peracetic acid (PAA; also referred to as peroxyacetic acid). Experiments were conducted in a pilot-scale 24 m³ stainless steel chamber using either biological indicators (Bis) or bacterial spores deposited onto surfaces via aerosolization. Wipe sampling was used to recover aerosol-deposited spores from chamber surfaces and coupon materials before and after fogging to assess decontamination efficacy. Temperature, relative humidity, and hydrogen peroxide vapor levels were measured during testing to characterize the fog environment. The fog completely inactivated all BIs in a test using a 60 mL solution of PAA (22% hydrogen peroxide/4.5% peracetic acid). In tests using aerosol-deposited bacterial spores, the majority of the post-fogging spore levels per sample were less than 1 log colony forming units, with a number of samples having no detectable spores. In terms of decontamination efficacy, a 4.78 log reduction of viable spores was achieved on wood and stainless steel. Fogging of PAA solutions shows potential as a relatively easy to use decontamination technology in the event of contamination with *B. anthracis* or other spore-forming infectious disease agents, although additional research is needed to enhance sporicidal efficacy.

Gayán et al. [67] evaluated the ability of UV light to inactivate bacterial spores of interest for food preservation and the effect of the absorptivity of the treatment medium on UV lethality and the effect of the simultaneous or sequential combination of UV light and mild heating. A UV dose of 23.72 J/mL reached 2.25, 2.93, 3.24, 3.85, and 4.05 log₁₀

reductions of *Bacillus coagulans*, *B. cereus*, *Alicyclobacillus acidocaldarius*, *Bacillus licheniformis*, and *G. stearothermophilus* spores, respectively. The most UV-sensitive species (*G. stearothermophilus*) was the most heat-resistant species. The shoulder phase of UV survival curves of the most resistant *B. coagulans* increased linearly with the absorptivity of the treatment medium whereas the inactivation rate decreased exponentially. The UV inactivation of *B. coagulans* increased synergistically when increasing the treatment temperature from 25 to 60 °C. A previous heat treatment (60 °C for 3.58 min) did not affect UV sensitivity of *B. coagulans*, but prior UV exposure (27.10 J/mL) sensitized bacterial spores to subsequent heat treatments. The authors concluded, unlike most non-thermal technologies, UV light is a real alternative to current heat sterilization treatments aimed at the inactivation of bacterial spores. The main limitation of UV bacterial inactivation is the absorption coefficient of the treatment medium, but this limitation is of similar importance in the pasteurization (inactivation of vegetative cells) and sterilization (inactivation of bacterial spores) processes. For UV sterilization of liquid foods with high absorption coefficients, the efficacy of UV light can be enhanced when combined with heat.

Banerjee et al. [68] studied porphyrin-based formulations to inactivate *Bacillus* spores and the effect of porphyrins alone and in combination with germinants against both *B. cereus* and *B. anthracis* spores in the presence of light. The authors tested the effect of two different porphyrins, aminemodified protoporphyrin IX (PPIX) and meso-tetra (N-methyl-4-pyridyl) porphine tetra tosylate (TMP). Treatment with the porphyrins alone did not significantly influence spore viability. However, when spores were pretreated with a solution containing the germinants, L-alanine and inosine, the spore viability dropped by as much as 4.5 logs in the presence of light. The extent of inactivation depended on the germination conditions and the type of porphyrin used, with TMP being more effective. The conclusion was the porphyrins can be used effectively in combination with germinants to inactivate *Bacillus* spores. According to this study, the results provide evidence that porphyrins can be used to inactivate *Bacillus* spores in the presence of germinants and light irradiation. This finding may be general and may be extended to spores of other pathogens.

March et al. [69] compared the sensitivity of spores from virulent and attenuated *B. anthracis* strains in suspension to inactivation by various chemical disinfectants. Spore suspensions from two virulent strains (A0256 and A0372) and two attenuated strains (Sterne and A0141) of *B. anthracis* were tested against two aldehyde-based disinfectants and one hypochlorite-based disinfectant. A novel statistical model was used to estimate 4 log₁₀ reduction times for each disinfectant/strain combination. Reduction times were compared statistically using approximate *Z* and χ^2 tests. Although there was no consistent correlation between virulence and increased sporicidal resistance across all three disinfectants, spores from the two virulent and two attenuated strains did display significantly different susceptibilities to different disinfectants. Significant disinfectant–strain interactions were observed for two of the three disinfectants evaluated. The comparative results suggest that the use of surrogate organisms to model the inactivation kinetics of virulent *B. anthracis* spores may be misleading. The accuracy of such extrapolations is disinfectant dependent and must be used with caution.

3. Conclusion

This chapter sought to provide the reader with a description of some methods of destroying spores of *Bacillus* spp. (sterilization/desinfection). The high level of resistance of spores caused serious problems in contamination of buildings that have suffered attacks of bioterrorism, and even today, deserves considerable attention in the food industry and in hospitals. Consequently, it is of significant interest to understand efficient methods of destruction or inactivation of spores.

Acknowledgements The support by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) for the scholarship provided (Process 2010/14434-4) is gratefully acknowledged.

References

- [1] Russell AD. The bacterial spore and chemical sporicidal agents. *Clin Microbiol Rev.* 1990;3:99-119.
- [2] McDonnell G, Russell AD. Antiseptics and disinfectants: activity, action and resistance. *Clin Microbiol Rev.* 1999;12:147-179.
- [3] Nicholson WL, Munakata N, Horneck G, Melosh HJ, Setlow P. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiol Mol Biol Rev.* 2000;64:548-572.
- [4] Setlow P, ed. *Bacterial stress responses*. Washington, DC: American Society for Microbiology; 2000.
- [5] Oliveira A, Almeida A, Carvalho CM, Tomé JP, Faustino MA, Neves MG, et al. Porphyrin derivatives as photosensitizers for the inactivation of *Bacillus cereus* endospores. *J Appl Microbiol.* 2009;106:1986-1995.
- [6] Schäfer M, Schmitz C, Facius R, Horneck G, Milow B, Funken KH, et al. Systematic study of parameters influencing the action of rose bengal with visible light on bacterial cells: comparison between the biological effect and singlet-oxygen production. *Photochem Photobiol.* 2000;71:514-523.
- [7] Crielly EM, Logan NA, Anderton A. Studies on the *Bacillus* flora of milk and milk products. *J Appl Bacteriol.* 1994;77:256-263.
- [8] Hammer P, Lembke F, Suhren G, Heeschen W. Characterization of a heat resistant mesophilic *Bacillus* species affecting quality of UHT-milk e a preliminary report. *Kieler Milchw Forsch.* 1995;47:303-311.

- [9] Klijn N, Herman L, Langeveld L, Vaerewijck M, Wagendorp AA, Huemer I, et al. Genotypical and phenotypical characterization of *Bacillus sporothermodurans* strains, surviving UHT sterilization. *Int Dairy J.* 1997;7:421-428.
- [10] Young SB, Setlow P. Mechanisms of killing of *Bacillus subtilis* spores by Decon and Oxone, two general decontaminants for biological agents. *J Appl Microbiol.* 2004;96:289-301.
- [11] Demidova TN, Hamblin MR. Photodynamic inactivation of *Bacillus* spores, mediated by phenothiazinium dyes. *Appl Environ Microbiol.* 2005;71:6918-6925.
- [12] Setlow P. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *J Appl Microbiol.* 2006;101:514-525.
- [13] Setlow B, Loshon CA, Genest PC, Cowan AE, Setlow C, Setlow P. Mechanisms of killing spores of *Bacillus subtilis* by acid, alkali and ethanol. *J Appl Microbiol.* 2002;92:362-375.
- [14] Sagripanti JL, Bonifacio A. Comparative sporicidal effects of liquid chemical agents. *Appl Environ Microbiol.* 1996;62:545-551.
- [15] Russell AD. Similarities and differences in the responses of microorganisms to biocides. *J Antimicrob Chemother.* 2003;52:750-763.
- [16] Whitney EA, Beatty ME, Taylor TH Jr, Weyant R, Sobel J, Arduino MJ, et al. Inactivation of *Bacillus anthracis* spores. *Emerg Infect Dis.* 2003;9:623-627.
- [17] Driks A, ed. *Bacillus subtilis and its closest relatives: from genes to cells.* Washington, DC: American Society for Microbiology; 2002.
- [18] Lai EM, Phadke ND, Kachman MT, Giorno R, Vazquez S, Vazquez JA, et al. Proteomic analysis of the spore coats of *Bacillus subtilis* and *Bacillus anthracis*. *J Bacteriol.* 2003;185:1443-1454.
- [19] Redmond C, Baillie LW, Hibbs S, Moir AJ, Moir A. Identification of proteins in the exosporium of *Bacillus anthracis*. *Microbiology.* 2004;150:355-363.
- [20] Waller LN, Fox N, Fox KF, Fox A, Price RL. Ruthenium red staining for ultrastructural visualization of a glycoprotein layer surrounding the spore of *Bacillus anthracis* and *Bacillus subtilis*. *J Microbiol Methods.* 2004;58:23-30.
- [21] Todd SJ, Moir AJ, Johnson MJ, Moir A. Genes of *Bacillus cereus* and *Bacillus anthracis* encoding proteins of the exosporium. *J Bacteriol.* 2003;185:3373-3378.
- [22] Daubenspeck JM, Zeng H, Chen P, Dong S, Steichen CT, Krishna NR, et al. Novel oligosaccharide chains of the collagen-like region of BelA, the major glycoprotein of the *Bacillus anthracis* exosporium. *J Biol Chem.* 2004;279:30945-30953.
- [23] Popham DL. Specialized peptidoglycan of the bacterial endospore: the inner wall of the lockbox. *Cell Mol Life Sci.* 2002;59:426-433.
- [24] Setlow P. Spore germination. *Curr Opin Microbiol.* 2003;6:550-556.
- [25] Cortezzo DE, Setlow P. Analysis of factors influencing the sensitivity of spores of *Bacillus subtilis* to DNA damaging chemicals. *J Appl Microbiol.* 2005;98:606-617.
- [26] Cowan AE, Olivastro EM, Koppel DE, Loshon CA, Setlow B, Setlow P. Lipids in the inner membrane of dormant spores of *Bacillus* species are immobile. *Proc Natl Acad Sci U S A.* 2004;101:7733-7738.
- [27] Gerhardt P, Marquis RE. Spore thermoresistance mechanisms. In: Smith I, Slepecky RA, Setlow P, eds. *Regulation of prokaryotic development.* Washington, DC: American Society for Microbiology; 1989:43-63.
- [28] Paidhungat M, Setlow P, eds. *Spore germination and outgrowth. Bacillus subtilis and its closest relatives: from genes to cells.* Washington, DC: American Society for Microbiology; 2002.
- [29] Tennen R, Setlow B, Davis KL, Loshon CA, Setlow P. Mechanisms of killing of spores of *Bacillus subtilis* by iodine, glutaraldehyde and nitrous acid. *J Appl Microbiol.* 2000;89:330-338.
- [30] Rutala WA, Weber DJ. Healthcare Infection Control Practices Advisory Committee. Guideline for Disinfection and Sterilization. In: *Healthcare Facilities.* Atlanta, GA: Department of Health and Human Services, Centers for Disease Control and Prevention; 2008.
- [31] Molins RA, ed. *Food irradiation: principles and applications.* New York, NY: Wiley-IEEE; 2001.
- [32] Hayashi N, Akiyoshi Y, Kobayashi Y, Kanda K, Ohshima K, Goto M. Inactivation characteristics of *Bacillus thuringiensis* spore in liquid using atmospheric torch plasma using oxygen. *Vacuum.* 2013;88:173-176.
- [33] Heinz V, Buckow R. Food preservation by high pressure. *J Cons Protect Food Safe.* 2010;5:73-81.
- [34] Nakayama A, Yano Y, Kobayashi S, Ishikawa M, Sakai K. Comparison of pressure resistances of spores of six *Bacillus* strains with their heat resistances. *Appl Environ Microbiol.* 1996;62:3897-3900.
- [35] Aouadhi C, Simonin H, Prévost H, Lamballerie M, Maaroufi A, Mejri S. Inactivation of *Bacillus sporothermodurans* LTIS27 spores by high hydrostatic pressure and moderate heat studied by response surface methodology. *LWT-Food Sci Technol.* 2013;50:50-56.
- [36] Garcia ML, Burgos J, Sanz B, Ordoñez JA. Effect of heat and ultrasonic waves on the survival of two strains of *Bacillus subtilis*. *J Appl Bacteriol.* 1989;67:619-628.
- [37] Ordoñez JA, Aguilera, MA, Garcia, ML, Sanz B. Effect of combined ultrasonic and heat treatment (thermoultrasonication) on the survival of a strain of *Staphylococcus aureus*. *J Dairy Res.* 1987;54:61-67.
- [38] Butz P, Tauscher B. Emerging technologies: chemical aspects. *Food Res Int.* 2002;35:279-284.
- [39] Scouten AJ, Beuchat LR. Combined effects of chemical, heat and ultrasound treatments to kill *Salmonella* and *Escherichia coli* O157:H7 on alfalfa seeds. *J Appl Microbiol.* 2002;92:668-674.
- [40] Seymour IJ, Burfoot D, Smith RL, Cox LA, Lockwood A. Ultrasound decontamination of minimally processed fruits and vegetables. *Int J Food Sci Tech.* 2002;37:547-557.
- [41] Ahmed AH, Moustafa MK, Marth EH. Incidence of *Bacillus cereus* in milk and some milk products. *J Food Protect.* 1983;46:126-128.
- [42] Kim HU, Goepfert JM. Occurrence of *Bacillus cereus* in selected dry food products. *J Milk Food Tech.* 1971;34:12-15.
- [43] Harmon SM, Kautter DA. Incidence and growth potential of *Bacillus cereus* in ready to serve foods. *J Food Protect.* 1991;54:372-374.

- [44] Kaneko K, Hayashidani H, Ohtomo Y, Kosuge J, Kato M, Takahashi K, et al. Bacterial contamination of ready-to-eat foods and fresh products in retail shops and food factories. *J Food Protect.* 1999;62:644-649.
- [45] King Jr AD, Magnusson JA, Török T, Goodman N. Microbial flora and storage quality of partially processed lettuce. *J Food Sci.* 1991;56:459-461.
- [46] Roberts D, Watson GN, Gilbert RJ. Contamination of food plants and plant products with bacteria of public health significance. In: Rhodes-Roberts ME, Skinner, FA, eds. *Bacteria and Plants*. London, UK: Society for Applied Bacteriology Symposium Series; 1982:169-195.
- [47] Russell A. Principles of antimicrobial activity and resistance. In: Block S, ed. *Disinfection, Sterilization and Preservation*. Philadelphia, PA: Lippincott Williams and Wilkins; 2001:31-57.
- [48] Setlow B, Setlow P. Binding of small, acid-soluble spore proteins to DNA plays a significant role in the resistance of *Bacillus subtilis* spores to hydrogen peroxide. *Appl Environ Microbiol.* 1993;59:3418-3423.
- [49] Setlow P. Mechanisms for the prevention of damage to DNA in spores of *Bacillus* species. *Annu Rev Microbiol.* 1995;49:29-54.
- [50] Sagong HG, Cheon HL, Kim SO, Lee SY, Park KH, Chung MS, et al. Combined effects of ultrasound and surfactants to reduce *Bacillus cereus* spores on lettuce and carrots. *Int J Food Microbiol.* 2013;160:367-372.
- [51] Blakistone B, Chuyate R, Kautter D, Charbonneau J, Suit K. Efficacy of oxonia active against selected spore formers. *J Food Prot.* 1999;62:262-267.
- [52] Khadre MA, Yousef AE. Sporicidal action of ozone and hydrogen peroxide: A comparative study. *Int J Food Microbiol.* 2001;71:131-138.
- [53] Langsrud S, Baardsen B, Sundheim G. Potentiation of the lethal effect of peroxygen on *Bacillus cereus* spores by alkali and enzyme wash. *Int J Food Microbiol.* 2000;56:81-86.
- [54] Pinna A, Sechi LA, Zanetti S, Usai D, Delogu G, Cappuccinelli P, Carta F. *Bacillus cereus* keratitis associated with contact lens wear. *Ophthalmology.* 2001;108:1830-1834.
- [55] Sudhaus N, Pina-Pérez MC, Martínez A, Klein G. Inactivation kinetics of spores of *Bacillus cereus* strains treated by a peracetic acid-based disinfectant at different concentrations and temperatures. *Foodborne Pathog Dis.* 2012;9:442-452.
- [56] Rice EW, Adcock NJ, Sivaganesan M, Rose LJ. Inactivation of spores of *Bacillus anthracis* Sterne, *Bacillus cereus*, and *Bacillus thuringiensis* subsp. *israelensis* by chlorination. *Appl Environ Microbiol.* 2005;71:5587-5589.
- [57] Delcomyn CA, Bushway KE, Henley MV. Inactivation of biological agents using oxone-chloride solutions. *Environ Sci Technol.* 2006;40:2759-2764.
- [58] Melly E, Setlow P. Heat shock proteins do not influence wet heat resistance of *Bacillus* spores. *J Bacteriol.* 2001;183:779-784.
- [59] Prasad GK, Ramacharyulu PV, Merwyn S, Agarwal GS, Srivastava AR, Singh B, et al. Photocatalytic inactivation of spores of *Bacillus anthracis* using titania nanomaterials. *J Hazard Mater.* 2011;185:977-982.
- [60] Tang CH. Effect of thermal pretreatment of raw soymilk on the gel strength and microstructure of tofu induced by microbial transglutaminase. *LWT-Food Sci Technol.* 2007;40:1403-1409.
- [61] Anderson AK, Finkelstein R. A study of the electro pure process of treating milk. *J Dairy Sci.* 1919;2:374-406.
- [62] Somavat R, Mohamed HM, Chung YK, Yousef AE, Sastry SK. Accelerated inactivation of *Geobacillus stearothermophilus* spores by ohmic heating. *J Food Eng.* 2012;108:69-76.
- [63] Uemura K, Takahashi C, Kobayashi I. Inactivation of *Bacillus subtilis* spores in soybean milk by radio-frequency flash heating. *J Food Eng.* 2010;100:622-626.
- [64] Sastry SK, Barach JT. Ohmic and inductive heating. *J Food Sci.* 2000;65:42-46.
- [65] Iciek J, Papiewska A, Molska M. Inactivation of *Bacillus stearothermophilus* spores during thermal processing. *J Food Eng.* 2006;77:406-410.
- [66] Wood JP, Calfee MW, Clayton M, Griffin-Gatchalian N, Touati A, Egler K. Evaluation of peracetic acid fog for the inactivation of *Bacillus anthracis* spore surrogates in a large decontamination chamber. *J Hazard Mater.* 2013;250-251:61-67.
- [67] Gayán E, Álvarez I, Condón S. Inactivation of bacterial spores by UV-C light. *Innov Food Sci Emerg Technol.* 2013. doi: 10.1016/j.ifset.2013.04.007.
- [68] Banerjee I, Mehta KK, Dordick JS, Kane RS. Light-activated porphyrin-based formulations to inactivate bacterial spores. *J Appl Microbiol.* 2012;113:1461-1467.
- [69] March JK, Cohen MN, Lindsey JM, Millar DA, Lowe CW, Bunnell AJ, et al. The differential susceptibility of spores from virulent and attenuated *Bacillus anthracis* strains to aldehyde- and hypochlorite-based disinfectants. *Microbiologyopen.* 2012;1:407-414.