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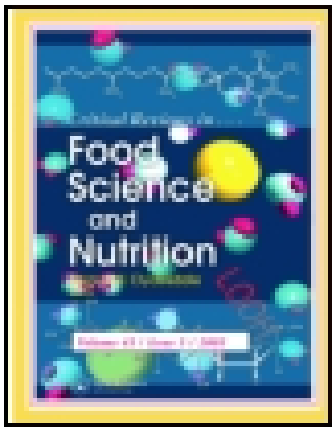

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Effect of High-pressure CO_2 Processing on Bacterial Spores

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Effect of High-Pressure CO_2 Processing on Bacterial Spores

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High-pressure CO_2 (HPCD) is a non-thermal technology that can effectively inactivate the vegetative forms of pathogenic and spoilage bacteria, yeasts, and molds at pressures less than 30 MPa and temperatures in the range of 20ºC to 40ºC. However, HPCD alone at moderate temperatures (20-40ºC) is often insufficient to obtain a substantial reduction in bacterial spore counts because their structures are more complex than those of vegetative cells. In this review, we first thoroughly summarized and discussed the inactivation effect of HPCD treatment on bacterial spores. We then presented and discussed the kinetics by which bacterial spores are inactivated by HPCD treatment. We also summarized hypotheses drawn by different researchers to explain the mechanisms of spore inactivation by HPCD treatment. We then summarized the current research status and future challenges of spore inactivation by HPCD treatment.

Key words HPCD, bacterial spore inactivation, inactivation kinetics, inactivation mechanism

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INTRODUCTION

Spores (or endospores) are highly resistant dormant forms of various bacteria, and the sporulation of vegetative cells occurs under harsh environments, such as poor nutrition. Spores are highly resistant to heat, chemicals, radiation, extreme temperatures (steam at 121ºC), UV

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radiation, and highly oxidative chemicals, e.g., ethylene oxide and hydrogen peroxide, which are used for sterilization (Setlow, 2006). The extreme resistance of bacterial spores to physical and chemical treatments makes them a significant problem in the food processing industry. Spores in food, which are typically from species of *Bacillus* and *Clostridium*, are common agents that cause spoilage, foodborne illnesses, and detrimental changes to the organoleptic quality (Brown, 2000). The major bacterial spore-forming foodborne pathogens are shown in Table 1.

Thermal processing is a well-known traditional technique that is used to reduce the microbial count of foods. Thermal processing at temperatures below 100ºC is defined as a disinfection process that destroys disease-causing and other harmful microorganisms but does not kill bacterial spores (Block, 2001; Russell, 2001). In contrast, thermal processing at a temperature of 121ºC or higher is a sterilization process that destroys all forms of life, including bacterial spores (Block, 2001; Russell, 2001). However, the high temperature required for thermal processing can impart undesirable organoleptic changes and cause some detrimental effects to the nutritional quality of heat-sensitive food. Due to the increased consumer demand for nutritious, fresh food products with a high organoleptic quality and an extended shelf life, non-thermal technologies (e.g., irradiation, pulsed electric fields, pulsed magnetic fields, high hydrostatic pressure, and high pressure CO_2) have been proposed as food-processing methods. Among the currently existing non-thermal technologies, the high hydrostatic pressure method is the most studied and is envisaged as a promising processing alternative for the improvement of the microbial safety of food products while preserving their nutritional and sensory characteristics (Devlieghere et al., 2004; Garcia-Gonzalez et al., 2007). Black et al. (2007) reviewed the response of bacterial spores to high hydrostatic pressure processing and indicated that a high hydrostatic pressure has the

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potential to inactivate bacterial spores. However, there are some drawbacks, such as the large investment cost due to the extremely high processing pressure and the non-continuous nature of the process, which hamper the industrial applications and commercialization of the high hydrostatic pressure methodology (Devlieghere et al., 2004; Estrada-Girón et al., 2005; Garcia-Gonzalez et al., 2007; Perrut, 2012).

High pressure CO_2 (HPCD) was first shown to inactivate *E. coli* cells in the 1950s by Fraster (1951). In the past 60 decades, the effects of HPCD on microorganisms have attracted the increasing attention of researchers. The use of HPCD as a sterilization method has several potential benefits. First, CO_2 is not flammable and is non-toxic, which means that HPCD processing is environmentally friendly and leaves no toxic residues. Second, the CO_2 pressures applied for sterilization are much lower (generally lower than 30 MPa) compared to the high pressures (100-600 MPa) employed in high hydrostatic pressure processing, which makes it easier to control and manage the pressure used in the HPCD technology. Third, the lower temperature used in the HPCD technology compared to thermal processing induces a much lower impact on the nutritional and physicochemical properties of food (Spilimbergo et al., 2010). The bactericidal effects of HPCD have been reviewed by Spilimbergo and Bertucco (2003), Balaban and Damar (2006), Zhang et al. (2006c), Garcia-Gonzalez et al. (2007), and Perrut (2011). These reviews mainly focused on the biocidal effect of HPCD on bacterial vegetative cells and contained limited discussion on the HPCD-induced inactivation of bacterial spores. Previous studies have indicated that the vegetative forms of pathogenic and spoilage bacteria, yeasts, and molds are completely inactivated by HPCD at pressures less than 30 MPa and temperatures in the range of 20 to 40ºC (Spilimbergo and Bertucco, 2003; Damar and

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Balaban, 2006; Zhang et al., 2006c; Perrut, 2012). However, the use of HPCD at moderate temperatures (20-40ºC) is often insufficient to obtain a substantial reduction in bacterial spore counts due to their more complex structure compared to vegetative cells (Spilimbergo and Bertucco, 2003; Damar and Balaban, 2006; Zhang et al., 2006c; Garcia-Gonzalez et al., 2007; Perrut, 2012). Therefore, HPCD has not yet delivered on its promise as a potential sporicide due to its inability to achieve industrial levels of sterilization. To demonstrate industrial-level sterilization for potential commercial application, it is essential to demonstrate at least a 6-log reduction in the number of bacterial spores (FDA, 1997; White et al., 2006; Zhang et al., 2006b; Perrut, 2012). In recent years, the number of published journal articles related to bacterial spore inactivation by HPCD treatment has significantly increased, reaching a cumulative number of 34 published journal articles in June 2012 (Figure 1). In 27 of these published articles, 12 species of bacterial spores have been investigated: *Bacillus subtilis* (Kamihira et al., 1987; Ishikawa et al., 1997; Ballestra and Cuq., 1998; Spilimbergo et al., 2002; Spilimbergo et al., 2003a; Watanabe et al., 2003a; Karajanagi et al., 2011), *B. cereus* (Ishikawa et al., 1997; Spilimbergo et al., 2003a; Watanabe et al., 2003a; Garcia-Gonzalez et al., 2009), *B. megaterium* (Ishikawa et al., 1997; Enomoto et al., 1997), *B. polymyxa* (Ishikawa et al., 1997), *B. coagulans* (Ishikawa et al., 1997; Furukawa et al., 2003; Watanabe et al., 2003a; Watanabe et al., 2003b; Furukawa et al., 2004; Furukawa et al., 2006), *B. licheniformis* (Furukawa et al., 2003; Watanabe et al., 2003a; Watanabe et al., 2003b; Furukawa et al., 2004; Furukawa et al., 2006), *B. pumilus* (Zhang et al., 2006a; Tarafa et al., 2009; Shieh et al., 2009; Checinska et al., 2011), *B. atrophaeus* (Hemmer et al., 2006; Zhang et al., 2006b; Qiu et al., 2009), *B. anthracis* (Zhang et al., 2007), *Geobacillus stearothermophilus* (Kamihira et al., 1987; Watanabe et al., 2003a; White et al., 2006; Hemmer

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et al., 2006; Furukawa et al., 2009), *Alicyclobacillus acidoterrestris* (Bae et al., 2009; Casas et al., 2012), and *Clostridium sporogenes* (Haas et al., 1989). As shown in Table 2, these published journal articles have shown that the HPCD technology was used in combination with other methods to kills the spores and that some of these combinations of achieved the required 6-log reduction in the number of bacterial spores.

SPORE STRUCTURE AND GERMINATION

Structure of Bacterial Spores

Spores exhibit great resistance to disinfection or sterilization procedures due to their unique structures (Driks, 1999; Madigan et al., 2002). A bacterial spore consists of the following structures (listed from outside to inside): an exosporium, a coat layer, an outer membrane, a cortex, a germ cell wall, an inner membrane, and a core (Leggett et al., 2012). The spore envelope (spore cortex, coat, and exosporium), which is the permeability barrier, is not only important in the spore resistance to pressure, chemical, and lytic enzyme attack but also for the establishment and maintenance of the spore dormancy by causing and maintaining a low water content in the central region (core) of the spore (Paidhungat et al., 2002; Paidhungat and Setlow 2002; Setlow 2003; Setlow, 2006). The inner membrane has an extremely low permeability to small molecules (Westphal et al., 2003; Cowan et al., 2004; Cortezzo et al., 2004; Cortezzo and Setlow, 2005). The low permeability of the inner membrane is important for protecting the spore DNA from DNA damaging chemicals (Cortezzo and Setlow, 2005). In contrast to the approximately 80% water content of the wet weight of vegetative cells, the spore core is highly dehydrated (approximately 28%-57% water content depending on the species), which makes it very resistant to moist heat (Beaman and Gerhardt, 1986). In addition, the Ca^{2+}-dipicolinic acid

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complex (Ca-DPA) and α/β-type small acid-soluble protein (SASP) that bind to DNA enhance the spore resistance to heat, oxidizing agents, and ultraviolet radiation (Slieman et al., 2001; Paidhungat and Setlow, 2002; Setlow, 2006).

Spore Germination as a Prerequisite of Spore Inactivation

With respect to spore inactivation, it is well established that spores have to be activated to germinate before being inactivated (Spilimbergo and Bertucco, 2003). Therefore, some researchers have suggested that spores are first activated and germinated and then inactivated during HPCD treatment (Ballestra and Cuq, 1998; Spilimbergo et al., 2002; Spilimbergo et al., 2003a; Watanabe et al., 2003b; Furukawa et al., 2004). Therefore, it is important to understand the process of spore germination, which is shown in Figure 2 (Setlow, 2003). Much of the work on spore germination to date has centered on the spores of *B. subtilis* due to the wealth of genetic information available for this species and the ease with which mutant strains can be generated (Kunst et al., 1997; Nicholson et al., 2000; Setlow, 2006). Therefore, *B. subtilis* spores were chosen as a representative model to illuminate the mechanism of spore germination. It has been established that fresh suspensions of some bacterial endospores will not (or only very slowly) germinate unless heated for some time (Evans and Curran, 1943). This process is known as heat activation. Spore activation can also be triggered by appropriate pH conditions or chemical exposure (Keynan and Evenchik, 1969). Although much work has been performed to determine the different aspects of heat activation (Murrell, 1961), the mechanism is not yet elucidated, and there is no theory that will account for all of the facts known about this phenomenon (Foster and Johnstone, 1990; Paidhungat and Setlow, 2002). Keynan et al. (1964) suggested that the heat activation phenomenon could be explained by assuming that heat or other agents change the

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tertiary structure of a protein responsible for the maintenance of the dormant state by reducing the disulfide linkages that stabilize the protein in a specific configuration. The partial denaturation of this protein is reversible by the reoxidation of the reduced disulfide bonds. Therefore, spore activation is a reversible process that breaks the dormant state of a spore and renders it poised to enter germination, but does not commit the spore to germination and outgrowth. Activated spores retain most properties of the dormant spore (Keynan and Evenchik, 1969). In contrast, once a spore is committed to germination, the spore can no longer return to its dormant state (Gould, 1969).

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The germination of spores is induced by nutrients (e.g., L-amino acids, D-sugars, and purine nucleosides) and a variety of non-nutrient agents (e.g., Ca-DPA, cationic surfactants, such as dodecylamine, lysozyme, high pressures, mechanical abrasion, and peptidoglycan fragments) (Figure 2) (Foster and Johnstone, 1990; Moir et al., 2002; Setlow, 2003; Moir, 2006; Shah et al., 2008; Leggett et al., 2012). Nutrient germinants bind to receptors in the spore's inner membrane, and this interaction triggers the release of the core's huge depot of Ca-DPA and cations, which are replaced with water (Setlow, 2003). These steps are termed Stage I of germination. At the end of Stage I, the spore has lost some of its resistance to moist heat due to the increased water content of the core (Setlow, 2003). The events in Stage I of germination trigger the progression into Stage II. The events in Stage I of germination activate the cortex lytic enzymes (CLEs) CwlJ and SleB, which are sufficient for cortex degradation (Makino and Moriyama, 2002; Chirakkal et al., 2002). The mechanism of SleB activation is not yet known. In contrast, CwlJ is most likely activated directly by the Ca-DPA released from the spore during Stage I (Paidhungat et al., 2001). The activated CLEs hydrolyze the cortex, and the core swells as a result of additional

water uptake and the expansion of the germ cell wall (Setlow et al., 2001). As a consequence, the proteins in the core become mobile, and the core enzymes begin to operate, which leads to the degradation of α/β-type SASP, the initiation of metabolism, and ultimately macromolecular synthesis (Nessi et al., 1998; Paidhungat and Setlow, 2002; Cowan et al., 2003). With the completion of Stage II of germination and the initiation of enzyme activity in the spore core, the spore germination process is completed and leads to spore outgrowth, which eventually converts the germinated spore into a growing cell. In addition to nutrients, there are a number of other agents that can trigger spore germination. High pressures either activate the germinant receptors (100-200 MPa) (Wuytack et al., 2000) or Ca-DPA release (500-600 MPa) (Paidhungat et al., 2002), whereas alkylamines, such as dodecylamine, directly trigger the release of Ca-DPA (Setlow et al., 2003). External Ca-DPA activates CwlJ, which results in cortex hydrolysis (Paidhungat and Setlow, 2000; Paidhungat et al., 2001). Lysozyme treatment also hydrolyses the cortex, but this process can be achieved only in spores with a defective coat that allows enzymes to penetrate into the cortex (Setlow, 2003). Mechanical abrasion appears to damage the spore cortex to some extent, which leads to the activation of either of the CLEs of the spore (Jones et al., 2005). Peptidoglycan fragments initiate the spore germination by binding to PrkC, which is a well-conserved, eukaryotic-like Ser/Thr membrane kinase localized in the inner membrane of *B. subtilis* spores (Shah et al., 2008).

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BACTERICIDAL EFFECTS OF HPCD ON BACTERIAL SPORES

Effect of Time, Pressure, and Temperature

Some researchers have achieved significant inactivation of bacterial spores by HPCD using a long treatment time (Hate et al., 1996; Enomoto et al., 1997; Spilimbergo et al., 2002;

Spilimbergo et al., 2003a). Hate et al. (1996) reported a 6-log reduction in the number of *B. subtilis* spores after HPCD at 20 MPa and 70ºC for 10 h. Enomoto et al. (1997) achieved an approximately 7-log reduction in the number of *B. megaterium* spores with HPCD treatment at 7.8 MPa and 60ºC for 50 h. Spilimbergo et al. (2002) reported a more than 7-log reduction in the number of *B. subtilis* spores after HPCD treatment at 7 MPa and 75ºC for 24 h. Spilimbergo et al. (2003a) observed a more than 7-log reduction in the number of *B. subtilis* spores after HPCD treatment at 9.0 MPa and 60ºC for 6 h. In general, the traditional moist heat sterilization time should not exceed 30 min (Killeen and McCourt, 2012). Therefore, the treatment duration used in these studies would be impractical for large-scale sterilization.

A greater inactivation of bacterial spores by HPCD was also achieved with a higher treatment pressure (Hata et al., 1996; Enomoto et al., 1997; Furukawa et al., 2004; Zhang et al., 2006a; Bae et al., 2009). Furukawa et al. (2004) observed that only 10% of *B. coagulans* spores and 20% of *B. licheniformis* spores were inactivated by HPCD treatment at 6.5 MPa and 35ºC for 120 min and subsequent heat treatment at 70ºC for 30 min. When the pressure was increased to 30 MPa, approximately 90% of the *B. coagulans* spores and 80% of the *B. licheniformis* spores were inactivated by HPCD treatment at 35ºC for 120 min and subsequent by heat treatment at 70ºC for 30 min (Furukawa et al., 2004). Zhang et al. (2006a) examined the effect of pressure on wetted spores of *B. pumilus* at 60ºC for 4 h and found that there was a significant decrease in the log-reduction achieved from 3.06 at 27.5 MPa to 1.91 at 10.3 MPa. As shown by Hata et al. (1996), the inactivation rate constant *k* for *B. subtilis* spores increases with increasing pressure (Figure 3), which means that a higher pressure could result in a greater inactivation of *B. subtilis* spores at constant treatment temperature and time. Similarly, Bae et al. (2009) investigated the survival

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curves of *A. acidoterrestris* spores in apple juice after HPCD treatment at different pressures (8 MPa, 10 MPa, and 12 MPa) for various times (10 min, 20 min, 30 min, and 40 min) at 65ºC and 70ºC and indicated that treatment at 12 MPa resulted in a greater inactivation of *A. acidoterrestris* spores than treatment at 8 or 10 MPa for less than 30 min. However, Enomoto et al. (1997) observed that the maximum bactericidal effect (approximately 7-log reduction) for inactivating *B. megaterium* spores by HPCD was approximately 5.8 MPa at 60ºC for 30 h and indicated that higher or lower pressures result in a decreased bactericidal effect. These authors attributed this phenomenon to a pressure-dependent clumping; this phenomenon will be discussed later in the review (Enomoto et al., 1997).

Some studies have suggested that the processing temperature plays a significant role in the inactivation of spores by HPCD. HPCD treatment at moderate temperature (lower than 60°C) is not sufficient for the inactivation of bacterial spores (Kamihira et al., 1987; Enomoto et al., 1997; Watanabe et al., 2003b). Most spores can be inactivated by a combination of HPCD and mild temperature treatments, although the effect depends on the spore species (Hate et al. 1996; Enomoto et al., 1997; Ballestra and Cuq, 1998; Spilimbergo et al., 2002; Watanabe et al., 2003b; Bae et al., 2009). Kamihira et al. (1987) did not observe any inactivation of *G. stearothermophilus* spores by HPCD and observed only a 53% inactivation of *B. subtilis* spores by HPCD at 20 MPa and 35ºC for 120 min. Garcia-Gonzalez et al. (2009) reported that only an approximately 1-log reduction in the number of *B. cereus* spores was achieved by HPCD at 10.5 MPa and 35ºC for 20 min. Enomoto et al. (1997) showed that there was no significant inactivation of *B. megaterium* spores by HPCD at temperatures less than 50ºC and that inactivation effect increased dramatically with increasing temperature from 50ºC to 60ºC.

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Moreover, an approximately 7-log reduction in the number of *B. megaterium* spores was achieved by HPCD treatment at 5.8 MPa and 60ºC for 30 h. Watanabe et al. (2003b) showed that there was no significant inactivation of *B. coagulans*, *B. cereus*, *B. licheniformis*, and *B. subtilis* spores after HPCD treatment at 30 MPa and 35ºC for 30-120 min. These researchers also found that HPCD at temperatures lower than 85ºC did not kill *G. stearothermophilus* spores, but a 5-log reduction in the number of *G. stearothermophilus* spores was achieved at 30 MPa for 120 min when the temperature was increased to 95ºC. Spilimbergo et al. (2002) reported that only a 0.5-log and a 0.9-log reduction in the number of *B. subtilis* spores could be achieved with HPCD treatments at 7.5 MPa and 36ºC for 24 h and at 12 MPa and 54ºC for 24 h, respectively. When the temperature was increased to 75ºC, a more than 7-log reduction in the number of *B. subtilis* spores was achieved at 7 MPa for 24 h. Similarly, Spilimbergo et al. (2003a) reported that a prolonged HPCD treatment (48 h) at a pressure up to 12 MPa and a temperature of 50ºC resulted in less than 1-log reduction in the number of *B. subtilis* spores. When the temperature was increased to 60ºC and 75ºC, a more than 7-log reduction in the number of *B. subtilis* spores was achieved at 9.0 MPa for 6 h and at 7.0 MPa for 2 h, respectively. In addition, Ballestra and Cuq (1998) observed an approximately 3.5-log reduction in the number of *B. subtilis* spores at 5 MPa and 80ºC for 60 min. Bae et al. (2009) achieved a more than 6-log reduction in the number of *A. acidoterrestris* spores suspended in apple juice by HPCD treatment at 10 MPa and 65ºC for 40 min and at 8 MPa and 70ºC for 30 min. Although these studies indicated that it is possible to significantly reduce the number of spores through a combination of heat and HPCD treatments, there were some drawbacks in the HPCD processing. The treatment time in these studies was too long for the development of a practical sterilization process (Hate et al., 1996; Enomoto et al.,

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1997; Spilimbergo et al., 2002), and the treatment temperatures were usually higher than 60ºC, which would be harmful to heat-sensitive foods (Hate et al., 1996; Ballestra and Cuq, 1998; Spilimbergo et al., 2002; Spilimbergo et al., 2003a; Watanabe et al., 2003).

Effect of the CO_2 State

Furukawa et al. (2003) investigated the effect of high-pressure gaseous CO_2 treatment (HGCT) at 35ºC and 6.5 MPa and high-pressure supercritical CO_2 treatment (HSCT) at 35ºC and 30 MPa (both treatments were followed by heat treatment at 70ºC) on *B. coagulans* and *B. licheniformis* spores. In both strains, HSCT was more effective for the inactivation of bacterial spores than HGCT. HGCT for 120 min resulted in approximately 10% of the *B. coagulans* spores being inactivated and 40% of the *B. coagulans* spores being germinated. Approximately 90% of the *B. coagulans* spores were inactivated and germinated after HSCT for 120 min. Moreover, HGCT for 120 min resulted in approximately 20% of the *B. licheniformis* spores being inactivated and 70% of the *B. licheniformis* spores being germinated, whereas HSCT for 120 min resulted in approximately 80% of the *B. licheniformis* spores being inactivated and 90% of the *B. licheniformis* spores being germinated. These results suggest that the spores germinated more effectively with HSCT than HGCT and that germinated spores are more sensitive to HSCT than HGCT. Hata et al. (1996) observed that the plots of the inactivation rate constant k as a function of the pressure are sigmoidal during the inactivation of *B. subtilis* spores by HPCD treatment, as shown in Figure 3. The values of k tended to increase definitely near the critical pressure of CO_2 (7.37 MPa) and increased gradually with increasing pressure above this critical pressure. Thus, HPCD should be performed at a pressure above the critical pressure of CO_2 for rapid sterilization. Therefore, it appears that supercritical CO_2 treatment is more effective for the

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inactivation of bacterial spores.

Effect of Cycled-Pressure and Microbubble Method

Several studies have indicated that the use of cycled pressure of microbubbles could enhance the inactivation of bacterial spores by HPCD treatment at a low temperature for a shorter duration (Spilimbergo et al., 2002; Spilimbergo et al., 2003a; Ishikawa et al., 1997). Spilimbergo et al. (2002) showed that a 3.5-log reduction in the number of *B. subtilis* spores could be achieved with HPCD treatment with pressure cycling (30 cycles/h, ΔP = 8 MPa) at 15 MPa and 36ºC for 30 min, whereas HPCD treatment with a lower pressure cycling (2-6 cycles/h, ΔP = 11 MPa) resulted in a 0.8~1.1-log reduction in the number of *B. subtilis* spores at 15 MPa and 54ºC for 60 min. In contrast, HPCD treatment at 7.5 MPa and 36ºC for 24 h without pressure cycling only resulted in a 0.5-log reduction. Similarly, Spilimbergo et al. (2003a) achieved a 2-log reduction in the number of *B. subtilis* spores by cycled-pressure HPCD treatment (20 cycles/h, ΔP = 8 MPa) at 15 MPa and 36ºC for 30 min, whereas HPCD treatment with a lower pressure cycling (2-6 cycles/h, ΔP = 11 MPa) resulted in a 0.8~1.1-log reduction in the number of *B. subtilis* spores at 15 MPa and 50ºC for 60 min. Based on these results, the inactivation of *B. subtilis* spores by cycled-pressure HPCD treatment was enhanced by increasing the number of cycles per hour. Ishikawa et al. (1997) found that *Bacillus* spores were effectively inactivated by HPCD treatment using the microbubble method, in which numerous CO_2 microbubbles are formed by feeding CO_2 through a filter at the bottom of a pressure chamber. According to the study performed by Ishikawa et al. (1997), a 6-log reduction in the number of *B. cereus*, *B. subtilis*, *B. megaterium*, *B. polymyxa*, and *B. coagulans* spores was achieved at 30 MPa and 45-55ºC for 60 min. These researchers also found that the microbubble HPCD treatment of *B. cereus*, *B. subtilis*,

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B. megaterium, *B. polymyxa*, and *B. coagulans* spores at 30 MPa and 40ºC for 30 min resulted in a greater reduction (approximately 3-log reduction) in the number of spores compared with a similar treatment without microbubbling. The increased inactivation ratio was likely attributed to an increase in the CO_2 concentration in the spore suspension and the enhanced amount of CO_2 absorbed by the spores (Kumugai et al., 1997) or to the pH effect caused by microbubble method (Perrut, 2012). However, few studies have investigated spore inactivation by HPCD treatment with cycled pressure or microbubbles Therefore, it is worthwhile to study pressure cycling and the microbubble method as promising methods that can be used to enhance bacterial spore inactivation at lower temperatures and with shorter treatment times.

Effect of the pH and a_w of the Treatment Media and the Water Content of Spores

The effectiveness of HPCD treatment for spore inactivation is also affected by the pH and a_w of the treatment media and the water content of the spores. The antimicrobial effect of HPCD treatment was synergistic at a lowered pH (Haas et al., 1989; Bae et al., 2009; Casas et al., 2012) and an increased water content in the spores (Kamihira et al., 1987; Zhang et al., 2006a) and antagonistic at a lowered a_w of the treatment media (Furukawa et al., 2009). Haas et al. (1989) lowered the pH of the media of *C. sporogenes* spores to 2.5, 3.0, and 4.0 and then treated the spore suspensions at 5.4 MPa and 70ºC for 120 min. The *C. sporogenes* spores suspended in media with pH values of 2.5 and 3.0 were completely inactivated, whereas the spores in a media with a pH value of 4.0 exhibited only a 0.8-log reduction. Bae et al. (2009) found that *A. acidoterrestris* spores suspended in apple juice (pH 3.47) were completely inactivated by HPCD treatment at 10 MPa and 65ºC for 40 min and at 8 MPa and 70ºC for 30 min. Casas et al. (2012) achieved a 6-log reduction in the number of *A. acidoterrestris* spores suspended in a citric acid

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solution with a pH value of 3.6 by HPCD treatment at 10 MPa and 30ºC for 30 min. These results indicate that HPCD treatment with a pH lower than 4 can effectively inactivate bacterial spores. It has been previously proposed that water is necessary to ensure that HPCD treatment exhibits a biocidal effect toward spores (Kamihira et al., 1987). Kamihira et al. (1987) reported that only 1% of dry (2-10% water content) *B. subtilis* spores were inactivated by HPCD treatment at 20 MPa and 35ºC for 2 h, whereas approximately 53% of wet (70-90% water content) *B. subtilis* spores were inactivated by HPCD treatment under the same conditions. Zhang et al. (2006a) observed that the addition of water to supercritical CO_2 resulted in a greater than 3-log reduction in the number of *B. pumilus* spores compared with HPCD treatment without addition of water at 27.5 MPa and 60ºC for 4 h. Furukawa et al. (2009) showed that the effects of HPCD treatment at 30 MPa and 95ºC for 120 min on the inactivation of *G. stearothermophilus* spores in the presence of sodium chloride (3% and 6% w/v) and glucose (6% and 12% w/v) were decreased in proportion to the solute concentration. These researchers attributed this protection to the decreased water activity caused by the addition of sodium chloride and glucose.

Effect of Antimicrobial Compounds

Another combination treatment was the addition of antimicrobial compounds to the CO_2 or samples before the HPCD treatment. The addition of even a low concentration of a strong oxidant, such as hydrogen peroxide, tert-butyl hydroperoxide, peracetic acid or trifluoroacetic acid, to CO_2 could achieve high-efficacy inactivation of bacterial spores at mild temperatures (35-60ºC) (White et al., 2006; Zhang et al., 2006a; Zhang et al., 2006b; Zhang et al., 2007; Hemmer et al., 2007; Tarafa et al., 2009; Shieh et al., 2009; Checinska et al., 2011). Zhang et al. (2006a) achieved 4~6.3-log reduction in the number of *B. pumilus* spores by HPCD treatment at

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27.5 MPa and 40-60ºC for 240 min with the addition of 70-200 ppm H_2O_2 to CO_2. Moreover, Zhang et al. (2006b) achieved a more than 6.25-log reduction in the number of *B. atrophaeus* spores by HPCD treatment at 27.5 MPa and 40ºC for 240 min with the addition of 200 ppm H_2O_2 to CO_2. Zhang et al. (2007) achieved a 5.74~6.14-log reduction in the number of *B. anthracis* spores by HPCD treatment at 27.5 MPa and 40ºC for 240 min with the addition of 200 ppm H_2O_2 to CO_2. Hemmer et al. (2007) achieved a more than 6-log reduction in the number of *G. stearothermophilus* and *B. atrophaeus* spores by HPCD treatment at 30 MPa and 40ºC for 60 min with the addition of 100 ppm H_2O_2 to CO_2. Tarafa et al. (2009) obtained an approximately 4.45~6.28-log reduction in the number of *B. pumilus* spores by HPCD treatment at 27.5 MPa and 60ºC for 240 min with the addition of 30% H_2O_2 to CO_2. In addition, Shieh et al. (2009) observed a 4-log reduction in the number of *B. pumilus* spores by HPCD treatment at 10 MPa and 50ºC for 45 min with the addition of 10% methanol containing either 12% H_2O_2 or 12% tert-butyl hydroperoxide or a mixture of 6% H_2O_2 and 6% tert-butyl hydroperoxide to CO_2. These researchers also achieved a 4~5-log reduction in the number of *B. pumilus* spores by HPCD treatment at 10 MPa and 50ºC for 45 min with the addition of 3.3% water containing 3% H_2O_2, 3.3% water containing 10% methanol and 0.5% formic acid, or 3.3% water containing 10% methanol, 1% formic acid, and 2% H_2O_2 to CO_2. Checinska et al. (2011) showed that a more than 6-log reduction in the number of *B. pumilus* spores can be achieved by HPCD treatment at 8-10 MPa and 50ºC for 15 min with the addition of 3.3% water and 0.1% H_2O_2 to CO_2. White et al. (2006) reported that a more than 6-log reduction in the number of *G. stearothermophilus* spores was achieved by HPCD treatment at 10.34 MPa and 60ºC for 60 min with the addition of trifluoroacetic acid or 5% peracetic acid to CO_2. Qiu et al. (2009) achieved a more than 6-log

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reduction in the number of *B. atrophaeus* spores by HPCD treatment at 10 MPa and 35-41ºC for 27 min with the addition of 55 ppm PAA to CO_2. All of these studies indicated that the addition of strong oxidants to CO_2 could result in a high inactivation ratio of bacterial spores at a lower temperature with a shorter treatment time. Other compounds as CO_2 additives, including methanol, ethanol, isopropyl alcohol, formic acid, acetic acid, malonic acid, succinic acid, citric acid, and phosphoric acid, were also investigated (Kamihira et al., 1987; White et al., 2006; Zhang et al., 2006a; Furukawa et al., 2009). However, the addition of these compounds resulted in limited inactivation in most cases. As shown by Furukawa et al. (2009), although the addition of ethanol to the spore suspension could decrease the water activity (as previously demonstrated, a low water activity protects spores from inactivation) of the suspending medium, this addition did not affect the inactivation of *G. stearothermophilus* spores by HPCD treatment. The authors explained that the anti-microbiological effect of ethanol can offset the protective effect caused by the addition of ethanol (Furukawa et al., 2009).

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Effect of Pulsed Electric Field

The combination of the HPCD and pulsed electric field treatments can efficiently inactivate bacterial spores. Spilimbergo et al. (2003b) reported that a partial inactivation (three orders of magnitude) of *B. cereus* spores was detected after the spores were subjected to a pretreatment of 25 kV/cm and 20 pulses and HPCD treatment at 20 MPa and 40ºC for 24 h. However, no inactivation was observed after HPCD treatment at 20 MPa and 40ºC for 24 h in the absence of the pulsed electric field pretreatment. The experimental results indicated a synergistic effect between the pulse electric field and the HPCD treatments. Although the combined HPCD and pulsed electric field treatments had the potential to reduce the number of bacterial spores in

media containing thermo-sensitive components, the required residence time was relatively long. Further studies are thus needed to investigate this promising combined treatment.

Effect of Spore Species and Clumping Phenomenon

Different species of spores exhibit different resistances to HPCD treatment. Watanabe et al. (2003b) investigated the inactivation kinetics of HPCD on bacterial spores from different species, including *B. coagulans*, *B. subtilis*, *B. cereus*, *B. licheniformis*, and *G. stearothermophilus*. The *D* values at 30 MPa are shown in Table 3. Based on the *D* values, the resistances of the spores of the five strains to HPCD are ranked as follows: *B. subtilis* > *G. stearothermophilus* > *B. licheniformis* > *B. coagulans* > *B. cereus.* In contrast, spore clumping could decrease the inactivation effect of HPCD treatment. Enomote et al. (1997) observed that there was an optimum pressure of 5.8 MPa in the subcritical region of CO_2 that exerted the maximum inactivation effects on *B. megaterium* spores and indicated that higher or lower pressures resulted in a decreased bactericidal effect. These authors hypothesized that a pressure-dependent aggregation (clumping) of the spores may occur and that this clumping phenomenon may inhibit the lethal effect of HPCD treatment. This phenomenon was later demonstrated by Furukawa et al. (2006). These researchers evidenced the clumping of *B. coagulans* and *B. licheniformis* spores during HPCD treatment at 6.5 and 30 MPa and 35ºC and determined that the clumping of both spore species increased with increasing treatment time. Furukawa et al. (2006) also found that the addition of a surfactant decreased the hydrophobicity of the spore surface and increased both the number of single spores and the rate of the inactivation ratio of *B. coagulans* and *B. licheniformis* spores.

INACTIVATION KINETICS OF HPCD TREATMENT OF BACTERIAL SPORES

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The development of inactivation kinetic models is important for understanding the inactivation effect, the role of the parameters, and prediction. Kinetic parameters that describe the course of inactivation, as well as the pressure and temperature dependence of the inactivation rate constants, are of key importance for the design and optimization of an HPCD sterilization process. Kinetic models are very important for the elucidation of the mechanism by which HPCD treatment inactivates spores. To date, only a few publications have proposed kinetic models, although an increased number of publications have reported that HPCD treatment can inactivate bacterial spores. The time course of isobaric and/or isothermal spore inactivation due to pressure and/or temperature can be described by first-order models (Hata et al., 1996; Enomote et al., 1997; Watanabe et al., 2003b; White et al., 2006; Qiu et al., 2009) and two-fraction models (Ballestra and Cuq, 1998).

The First-Order Kinetic Model

A first-order kinetic model is often used to describe the heat inactivation of bacterial spores, in which the amount of viable spores decreases linearly as a function of time (Abraham et al., 1990). This model can be mathematically described as follows:

$$\log_{10}\left(N / N_0\right) = -kt\,, \qquad (1)$$

where k is the inactivation rate constant [h^{-1}], N is the concentration of viable spores [colonies/mL], t is the inactivation time [h], and N_0 is N at t = 0 [colonies/mL] (N/N_0 is the survival ratio). The decimal reduction time for HPCD treatment (D value) is defined as the time required to achieve a 90% reduction in the number of bacterial spores in HPCD treatment experiments. The D value is calculated from the negative reciprocals of the slopes of the regression lines of the deactivation curves. The Z value is defined as the temperature required to

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decrease the D value by 10-fold as a result of HPCD treatment. The Z value is calculated by determining the negative reciprocals of the slopes of the D value curves (log D vs. temperature). The temperature dependence of the inactivation rate constant is expressed by an Arrhenius equation:

$$k = A\exp\left(-E_a / RT\right) \qquad (2)$$

or

$$\ln k = -E_a / RT + \ln A, \qquad (3)$$

where A is the frequency factor [h^{-1}], E_a is the activation energy for inactivation [J/mol], R is the gas constant [8.314 J/mol·K], and T is the absolute temperature [K]. The time required for the pressure to become constant in an inactivation experiment was defined as $t = 0$. The inactivation rate constant k can be obtained from the plot of $\log_{10}(N/N_0)$ as a function of t. The parameters A and E_a are calculated from the plot of ln k as a function of $1/T$.

Hata et al. (1996) studied the inactivation kinetics of HPCD treatment on *B. subtilis* spores and observed a linear relationship irrespective of the temperature and the pressure (Figure 3). This result indicates that the time course of the survival ratio can be described by first-order reaction kinetics. Enomote et al. (1997) examined the lethal effect of HPCD treatment on *B. megaterium* spores at various pressures and temperatures and for various treatment times. As shown in Figure 4(a), the linear relationship obtained indicates that the inactivation kinetics can be represented using a first-order model. Watanabe et al. (2003b) investigated the inactivation kinetics of HPCD on bacterial spores of different species, including *B. coagulans*, *B. subtilis*, *B. cereus*, *B. licheniformis*, and *G. stearothermophilus*, and showed that the bacterial spore survival curves

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followed first-order kinetics, as shown in Figure 4(b). White et al. (2006) reported that the inactivation kinetics of *G. stearothermophilus* spores by HPCD treatment is also described by a first-order model, as shown in Figure 4(c). Qiu et al. (2009) found that the inactivation kinetics of *B. atrophaeus* spores by a combination of HPCD and peracetic acid treatments could be described by the first-order model shown in Figure 4(d). Based on these first-order models of spore inactivation by HPCD treatment, the inactivation rate constant was found to be closely related to treatment pressure and temperature. In general, an increased pressure and/or temperature could result in an increased inactivation rate constant (hate et al., 1996). However, the spore clumping during HPCD treatment needs to be considered (Enomote et al., 1997; Furukawa et al., 2006). The spore resistance to HPCD is also dependent on the species. Watanabe et al. (2003b) reported that *B. subtilis* spores exhibited the most resistance to HPCD treatment, whereas *B. cereus* spores were the most sensitive to HPCD treatment. In addition, spores are more effectively inactivated by HPCD treatment than by heat treatment alone. Hata et al. (1996) reported that the activation energy of the inactivation of *B. subtilis* spores by HPCD treatment was 30-90 kJ/mol, which is 1/15-1/5 of that required for heat sterilization. Watanabe et al. (2003b) reported that the activation energy for the inactivation of *G. stearothermophilus* spores by HPCD treatment was 94.5 kJ/mol at 75-95ºC, whereas the activation energy for the inactivation of *G. stearothermophilus* spores by heat treatment was 351 kJ/mol. Therefore, less energy is needed for the inactivation of spores by HPCD treatment.

The Two-Fraction Kinetic Model

Ballestra and Cuq (1998) observed that the survival curve of *B. subtilis* spores during HPCD treatment at 5 MPa and 80ºC showed two apparent distinct stages, as illustrated in Figure 4(e).

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The inactivation rate was slow at first and then increased sharply. Although two stages of the survival curves during spore inactivation by HPCD treatment were observed, no detailed mathematical models have been developed.

INACTIVATION MECHANNISM

Some researchers have proposed a possible inactivation mechanism in which the spores are first activated and germinated and then inactivated during HPCD treatment (Ballestra and Cuq, 1998; Spilimbergo et al., 2002; Spilimbergo et al., 2003a; Watanabe et al., 2003b; Furukawa et al., 2004) (Figure 5). These researchers proposed this "germinated and inactivated mechanism" based on their kinetic studies of spore inactivation by HPCD treatment. Unfortunately, it was not known how HPCD treatment induced spores to germinate. Other researchers developed another possible hypothesis for spore inactivation by HPCD treatment, which stated that the spore structure was damaged such that spore death was induced (Spilimbergo et al., 2003b; Zhang et al., 2006a; Zhang et al., 2006b; Zhang et al., 2007; Bae et al., 2009; Perrut, 2012) (Figure 5). These researchers proposed this "structure damaged and inactivated mechanism" based on their morphological and molecular studies of spore inactivation by HPCD treatment.

Kinetic Process Mechanisms

Some studies have investigated the process to demonstrate the mechanism by which HPCD treatment results in spore inactivation. Ballestra and Cuq (1998) suggested that the inactivation included two steps based on the two phases in the inactivation kinetics of *B. subtilis* spores at 5 MPa and 80ºC. The first step represented the penetration of CO_2 into the spore cells concomitant with the heat activation of dormant spores. Heat activation leads to cell modifications, such as the activation of enzymes in spores (Sapru et al., 1992), which could make the spores more

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sensitive to the metabolic disturbance induced by a large amount of antimicrobial CO_2 in the cells. Then, CO_2 damages the cell structure and metabolic systems involved in spore germination, which results in inactivation. Watanabe et al. (2003b) reported that there were two distinct linear portions of the log *D* value plots and that there were two corresponding linear portions in the Arrhenius plots obtained from the inactivation of *G. stearothermophilus* spores by HPCD treatment at 30 MPa and 35-95ºC. The results were similar to those obtained at high hydrostatic pressure (Okazaki et al., 1994). In addition, dormant bacterial spores germinated under hydrostatic pressure during pressure treatment, and the germinated spores were inactivated (Clouston and Wills, 1969; Gould and Sale, 1970; Wuytack et al., 1998). Similar to the results obtained with the pressure treatment, bacterial spores germinate during HPCD treatment (Furukawa et al., 2004). Therefore, Watanabe et al. (2003b) indicated that the inactivation mechanism by which HPCD inactivates bacterial spores is likely similar to that induced by high hydrostatic pressure. Spilimbergo et al. proposed that a combined treatment of temperature (at least 60ºC) and CO_2 induces shock in the spore structure, which leads to their activation. Consequently, during a long contact time of HPCD treatment, the spores could start their germination. The geminated spores then become more sensitive to the antimicrobial effect of CO_2, which ultimately results in their inactivation (Spilimbergo and Bertucco, 2003; Spilimbergo et al., 2003a). Spilimbergo et al. (2002) observed a tyndallization effect (approximately 3.5-log reduction) in *B. subtilis* spores as a result of cycled-pressure (30 cycles/h, ΔP = 8 MPa) HPCD treatment at 15 MPa and 36ºC for 30 min. These researchers explained the inactivation mechanism as follows: the first pressure cycles induce spore activation such that germination takes place during the holding time between two different cycles. In the following cycles, after

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spore activation and germination, inactivation would be possible (Spilimbergo et al., 2002).

Morphological and Molecular Mechanisms

Some studies have investigated the changes in the spore structure and constituents that occurring HPCD treatment to illustrate the spore inactivation mechanism induced by HPCD treatment. To visually observe the structural changes in spores, scanning electron microscopy (SEM) (Spilimbergo et al., 2003b; Bae et al., 2009) and transmission electron microscopy (TEM) (Zhang et al., 2006b; Zhang et al., 2007; Bae et al., 2009) have been applied to observe the surface and internal structural changes induced by HPCD treatment. DPA (pyridine-2, 6-dicarboxylic acid) is released when spores are activated for germination or when the spore envelope (spore cortex, inner and outer coat, and exosporium) is damaged (Sonenshein et al., 2002). Thus, DPA is often used as a marker molecule to identify germination or damage to the spore envelope (Zhang et al., 2006b; Zhang et al., 2007).

Through SEM, Spilimbergo et al. (2003b) observed cracks or folds on the surface of *B. cereus* spores pretreated with a pulsed electric field at 25 kV/cm and 20 pulses and then subjected to HPCD treatment at 20 MPa and 40ºC for 24 h (Figure 6). In addition, the shape of the treated spores appeared less spherical compared with the control. The authors proposed two hypotheses to explain the inactivation mechanism (Spilimbergo et al., 2003b). One hypothesis suggested that *B. cereus* spores likely underwent shock during the pulsed electric field treatment and that this shock might induce spore germination, which would take place during the long residence time under the HPCD conditions (15-24 h); after this point, spore inactivation would be possible. The other hypothesis suggested that *B. cereus* spores likely underwent shock during the pulsed electric field treatment that partially stressed their structure and that the stressed structure of the

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spore became more vulnerable to the subsequent HPCD treatment. Zhang et al. (2006b) investigated the mechanism by which *B. atrophaeus* spores are inactivated by HPCD treatment through transmission electron microscopy (TEM) and DPA analysis. These researchers observed that the spores subjected to HPCD treatment (27.5 MPa and 40ºC for 4 h) in the presence of H_2O_2 (200 ppm) displayed damage to the external spore envelope and an apparent loss of integrity of the inner structure, which agreed with the high inactivation rate and the high DPA release observed. These authors suggested that the spore envelope was damaged by the combined treatment of HPCD and H_2O_2 (Figure 7), which allowed the penetration of H_2O_2 into the cell, the oxidation of some vital internal structures, and caused spore death (Zhang et al., 2006b). However, under the same HPCD treatment conditions (27.5 MPa and 40ºC for 4 h) with the addition of H_2O_2 (200 ppm), Zhang et al. (2007) achieved a more than 6-log reduction in the number of *B. anthracis* spores, even though only mild changes in the spore structure were observed (Figure 8). The differences between the results obtained by Zhang et al. (2006b) and Zhang et al. (2007) are likely due to the different spore species used in the studies. Further studies are thus needed to better elucidate the inactivation mechanism. Bae et al. (2009) used SEM and EF-TEM (energy-filtering transmission electron microscopy) methods to examine the structural and morphological changes that *A. acidoterrestris* spores undergo during HPCD treatment at 10 MPa and 70ºC for 30 min (Figure 9). The SEM images revealed that the treated spores were crushed and exhibited a high degree of hollowness on the surface. In the EF-TEM photographs, an enlarged periplasmic space and a loss of cytoplasm were observed in the treated spores. Based on these images, the authors concluded that HPCD treatment directly affects and inactivates *A. acidoterrestris* spores.

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FUTURE OUTLOOK

As described in this paper, some work has been performed during the two last decades to improve our understanding of the sporicidal effect of HPCD. However, there will still be many investigations and challenges in the future.

To date, 12 species of *Bacillus* and *Clostridial* spores have been processed by HPCD treatment, but only one species of *Clostridial* spores has been investigated. *Clostridial* spores are common agents that cause spoilage, foodborne illnesses, and detrimental changes to the organoleptic quality. Therefore, much more work need to be performed to investigate the effect of HPCD on *Clostridial* spores.

Watanabe et al. (2003b) studied five species of spores (*B. subtilis*, *G. stearothermophilus*, *B. licheniformis*, *B. coagulans* and *B. cereus*) and found that *B. subtilis* spores exhibit the most resistance to HPCD treatment; however, this evidence is not sufficient to conclude that *B. subtilis* spores are the most resistant spores to HPCD treatment of all of the species of bacterial spores that are related to food spoilage or foodborne illnesses. Therefore, a comprehensive and systematic study needs to be performed to identify the bacterial spore with the most resistance to HPCD treatment. This most resistant spore can then be used as a biological indicator to evaluate the effectiveness of different HPCD sterilization processes.

Although some researchers have achieved the complete inactivation of bacterial spores, the experimental conditions appear to be too extreme (e.g., high temperature and long treatment time). Pressure cycling, microbubbling, and the addition of a strong oxidant appear to be promising methods for the enhancement of the inactivation effect of HPCD treatment at a low temperature for a short treatment time. However, the literature on these methods is scarce, and

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the studies are not comprehensive. Thus, a key task in the future is to develop an optimal method to achieve sterilization under mild process conditions.

Although several mechanisms by which HPCD treatment inactivates spores have been proposed, it is difficult to determine which (if any) is correct due to the lack of abundant and unambiguous data. Thus, a much deeper and more comprehensive analysis needs to be performed to elucidate how HPCD treatment inactivates bacterial spores. Moreover, the mathematical modeling of HPCD sterilization needs to be strengthened to illuminate the mechanism and determine the optimal processing conditions.

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Table 1 Foodborne illnesses and spoilage caused by spore-forming bacteria

Spore-forming bacteria	Type of illnesses	Type of spoilage	Reference
Clostridium botulinum	Foodborne botulism		Brown, 2000; Lund, 1990
Clostridium perfringens	Acute diarrhea and severe abdominal pain		Brown, 2000
Bacillus licheniformis	Nausea, vomiting, diarrhea, and stomach cramps		Logan, 2011
Bacillus cereus	Diarrhea and emesis	Bitty cream and sweet curdling	Brown, 2000; Andersson et al., 1995
Bacillus subtilis	Vomit and diarrhea	Spoilage of pasteurized milk	Brown, 2000; Logan, 2011
Bacillus sporothermodurans		Spoilage of UHT milk	Westhoff and Dougherty, 1981
Geobacillus stearothermophilus		Flat-sour spoilage	Brown, 2000
Bacillus coagulans		Flat-sour spoilage	Brown, 2000
Clostridium sporogenes		Gas and putrefactive odor	Brown, 2000
Clostridium thermosaccharolyticum		Gas and cheesy odor	Brown, 2000
Clostridium butyricum		Gas and butyric odor	Dasgupta and Hull, 1989; Brown, 2000
Clostridium tyrobutyricum		Gas and butyric odor	Dasgupta and Hull, 1989
Clostridium putrefaciens		Spoilage of cooked ham	Roberts and Derrick, 1975; Brown, 2000
Alicyclobacillus acidoterrestris		Spoilage of fruit juices	Splittstoesser et al., 1994; Brown, 2000

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Table 2 Inactivation of bacterial spores by high-pressure CO_2

Treatment combination	Species	Suspending medium	Conditions	Conditions			Log reduction	References
				Pressure (MPa)	Temperature (ºC)	Time (min)		
HPCD + heat	*Geobacillus stearothermophilus* *Bacillus subtilis*	Physiological saline		20	35	120	0 0.3	Kamihira et al.,1987
	Bacillus subtilis	Physiological saline		7.4-20 7.5 12 7	40-54 36 54 75	30-90 1440 1440 1440	0.9-1.1 0.5 0.9 >7	Spilimbergo et al.,2002
	Bacillus subtilis	Peptone solution		20	60-90	120	0.5-6	Hata et al., 1996
	Bacillus subtilis	Sterile ringer solution		5	80	60	3.5	Ballestra and Cuq, 1998
	Bacillus megaterium	Sterile distilled water		5.9 5.7	60 60	1440 1800	5.8 7	Enomoto et al., 1997
	Bacillus subtilis	Physiological saline		7.5-12 7 9	36-50 75 60	1440-2880 120 360	0.5-1 7 7	Spilimbergo et al.,2003a
	Bacillus subtilis	Polyethylene glycol gel		7.5 15 5	70 70 80	360 240 60	7	Karajanagi et al., 2011
	Bacillus coagulans *Bacillus cereus* *Bacillus licheniformis* *Bacillus subtilis* *Geobacillus stearothermophilus*	Sterile distilled water		30	35 35 35 35 35-95	30-120 30-120 30-120 30-120 20-120	0.7-1.5 0.7-1.5 0.7-1.5 0.5 0.5-5	Watanabe et al.,2003b
	Bacillus cereus	Sterile distilled water		10.5	35	20	1	Garcia-Gonzalez et al., 2009
HPCD +	*Bacillus subtilis*	Physiological		15	36-54	30-60	0.8-3.5	Spilimbergo et al., 2002

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pressure cycling		saline						
	Bacillus subtilis	Physiological saline		15	36-50	30-60	0.8-2	Spilimbergo et al., 2003a
HPCD + acid environment	*Clostridium sporogenes*	Growth medium	pH 2.5 pH 3.0 pH 4.0	5.4	70	120	7.8 7.5 0.8	Haas et al., 1989
	Alicyclobacillus acidoterrestris	Apple juice	pH 3.47	10 8	65 70	40 30	>6	Bae et al., 2009
	Alicyclobacillus acidoterrestris	Apple cream Apple cream Sterile water Citric acid solution	pH 3.61 pH 3.68 pH 5.81 pH 3.60	10	60 30 30 30	30	4 4 5 6	Casas et al., 2012
HPCD + microbubble method	*Bacillus polymyxa* *Bacillus coagulans* *Bacillus cereus* *Bacillus subtilis* *Bacillus megaterium*	Physiological saline		30	45 40 50 55 40	60 30 60 60 30	6	Ishikawa et al., 1997
HPCD + pulsed electric field	*Bacillus cereus*	Sterile distilled water		20 30	40 40	900 1440	1.5 3	Spilimbergo et al., 2003b
HPCD + compounds	*Geobacillus stearothermophilus*	Sterile distilled water	2% ethanol 0.5% acetic	20	35	120	0.3	Kamihira et al., 1987
	Geobacillus stearothermophilus	Spore strips	Ethanol 50% Citric acid Succinic acid Phosphoric acid 50% H_2O_2 Formic acid Acetic acid Malonic acid TFA 5% PAA	10.34	50-60 60 50 50 50 50 50 50 60 60	180 120 120 180 60 120 120 120 60 60	1.2-4 0.03-0.62 0.25-0.29 0.18-0.25 0.13-1.57 0	White et al., 2006

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						0.12-0.85 0-0.12 >6.4 >6.4	
Bacillus pumilus	Spore strips	Water 70% ethanol 70% IPA 70-200ppmH_2O_2	27.5	50-80 40 40 40-60	240	0.6-3 0.3 0.2 4-6.3	Zhang et al., 2006a
Bacillus anthracis	Spore strips	200ppm H_2O_2	27.5	40	240	5.74-6.14	Zhang et al., 2007
Bacillus atrophaeus	Spore strips	200ppm H_2O_2	27.5	40	240	>6.25	Zhang et al., 2006b
Geobacillus stearothermophilus *Bacillus atrophaeus*	Spore strips	<100ppm H_2O_2	30	40	60	>6	Hemmer et al., 2006
Bacillus atrophaeus	Porcine acellular dermal matrix	55ppm PAA	10	35-41	27	>6	Qiu et al., 2009
Bacillus pumilus	Spore strips	30% H_2O_2	27.5	60	120-240	4.45-6.28	Tarafa et al., 2009
Bacillus pumilus	Physiological saline	H_2O_2 and/or t-TBHP	10	50	45	4	Shieh et al., 2009
Bacillus pumilus	Sterile deionized water	3.3% water and 0.1% H_2O_2	8-10	50	15	>6	Checinska et al., 2011

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Table 3 Parameters of spore inactivation kinetics by high-pressure CO_2

Organisms	Additive	Pressure (MPa)	Temperature (ºC)	*D* (min)	*Z* (ºC)	E (kJ/mol)	Reference
Geobacillus stearothermophilus		30	35	385			Watanabe et al., 2003b
			55	182	135	18.0	
			65	196			
			75	179			
			85	130	25.7	94.5	
			95	29.9			
Bacillus subtilis			35	1667			
Bacillus coagulans			35	164			
Bacillus cereus			35	133			
Bacillus licheniformis			35	182			
Bacillus subtilis		4-20	60-90			30-90	Hata et al., 1996
Geobacillus stearothermophilus	0.002% peracetic acid	9.6	35	3.25			White et al., 2006
Bacillus atrophaeus	55 ppm peracetic acid	10	35-41	6.3			Qiu et al., 2009

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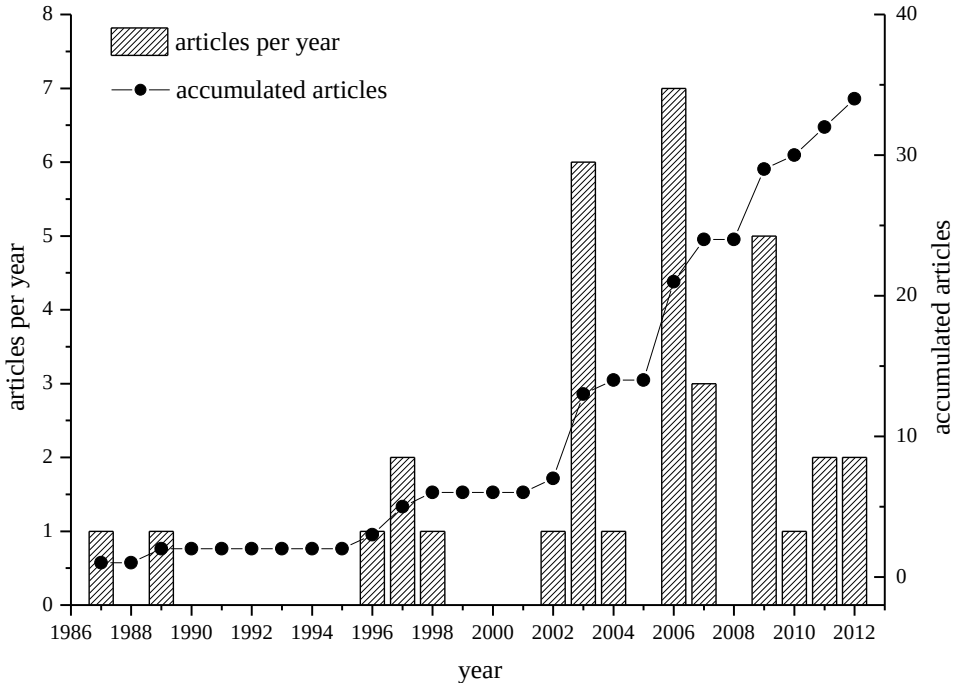

Figure 1 Published journal articles (bars) and cumulative number of articles (line) per year on the high-pressure CO_2- induced inactivation of bacterial spores. The articles taken into account were retrieved from different online resources (e.g., ISI Web of Knowledge and Elsevier ScienceDirect).

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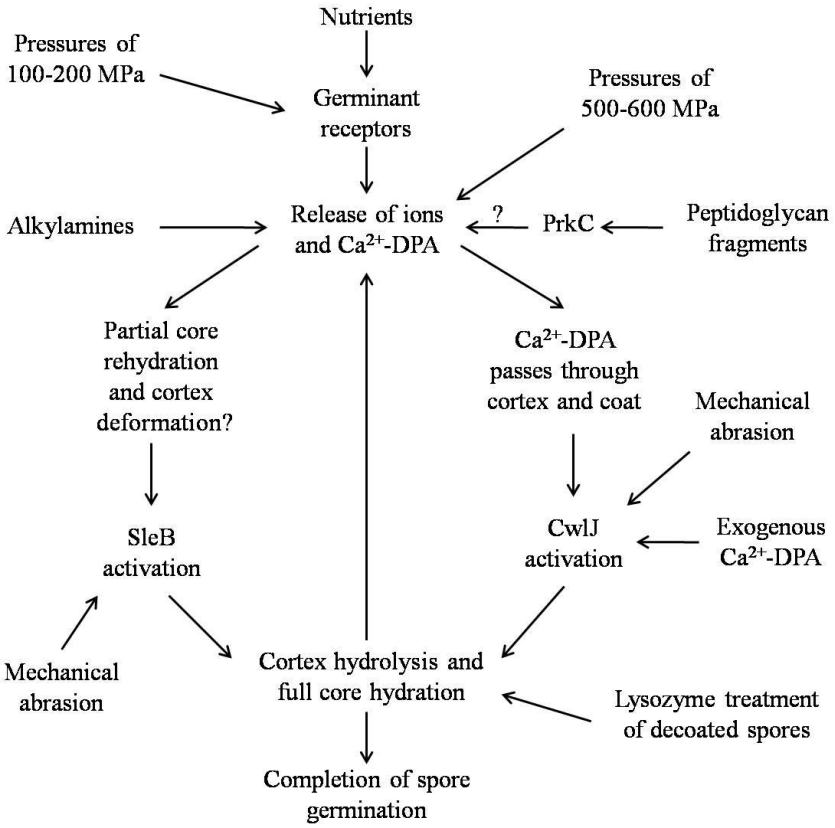

Figure 2 Model of nutrient and non-nutrient germination of *Bacillus subtilis* spores (adapted from Setlow, 2003).

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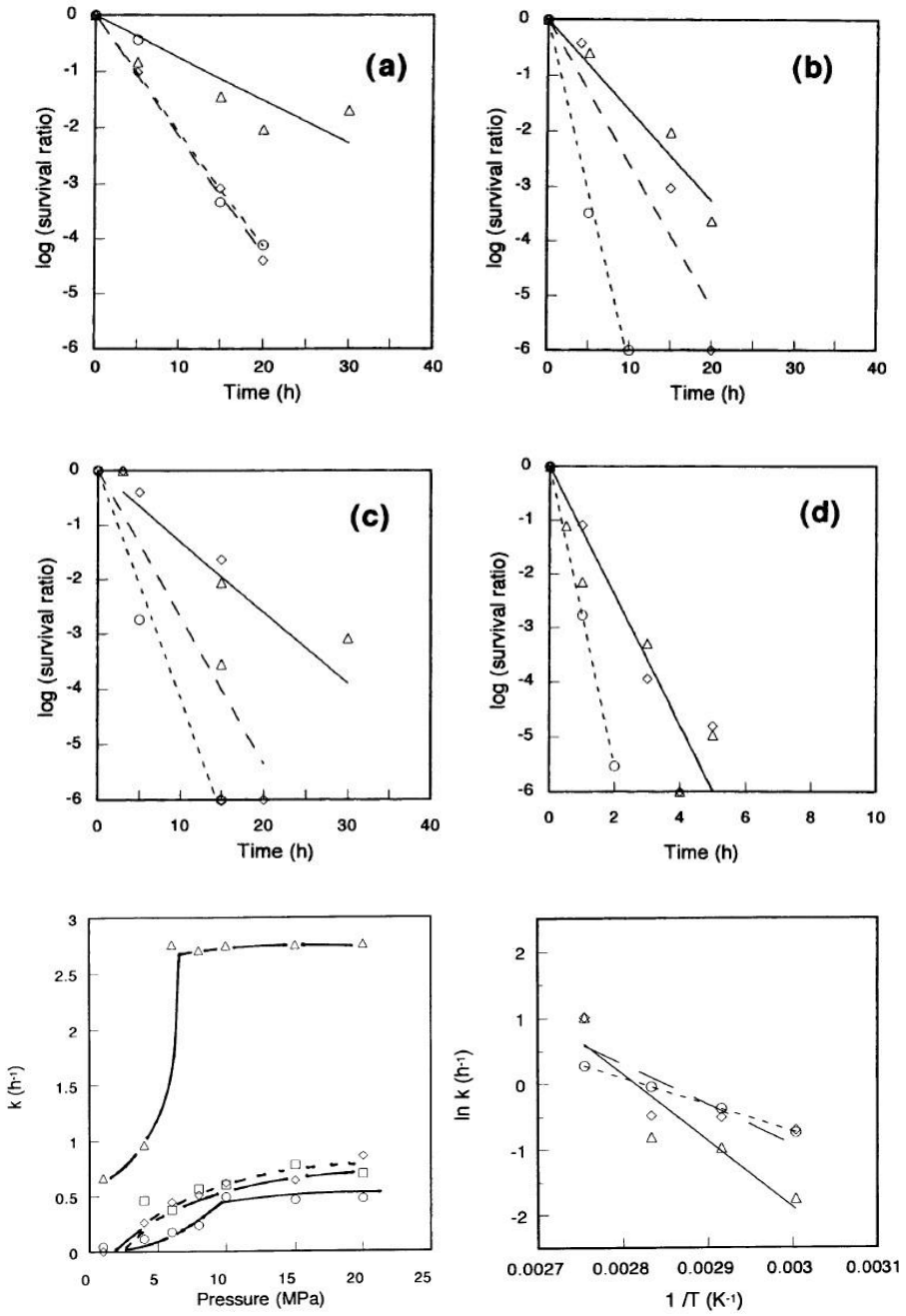

Figure 3 Kinetics of the inactivation of *Bacillus subtilis* spores by high-pressure CO_2 (from Hata et al., 1996).

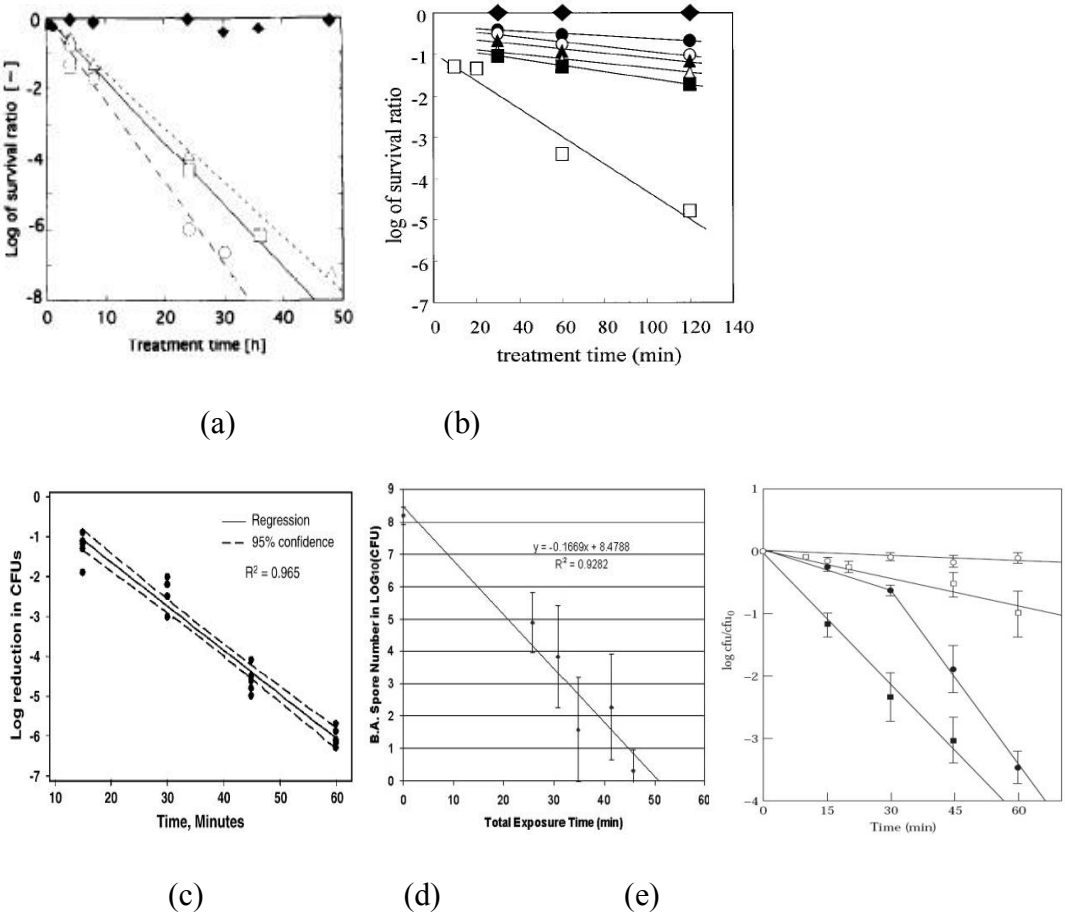

Figure 4 Kinetics of the inactivation of *Bacillus* spores by high-pressure CO_2: (a) *Bacillus megaterium* spores (from Enomote et al., 1997); (b) *Bacillus coagulans*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis*, and *Geobacillus stearothermophilus* spores (from Watanabe et al., 2003b); (c) *Geobacillus stearothermophilus* spores (from White et al., 2006); (d) *Bacillus atrophaeus* spores (from Qiu et al., 2009); and (e) *Bacillus subtilis* spores (from Ballestra and Cuq, 1998).

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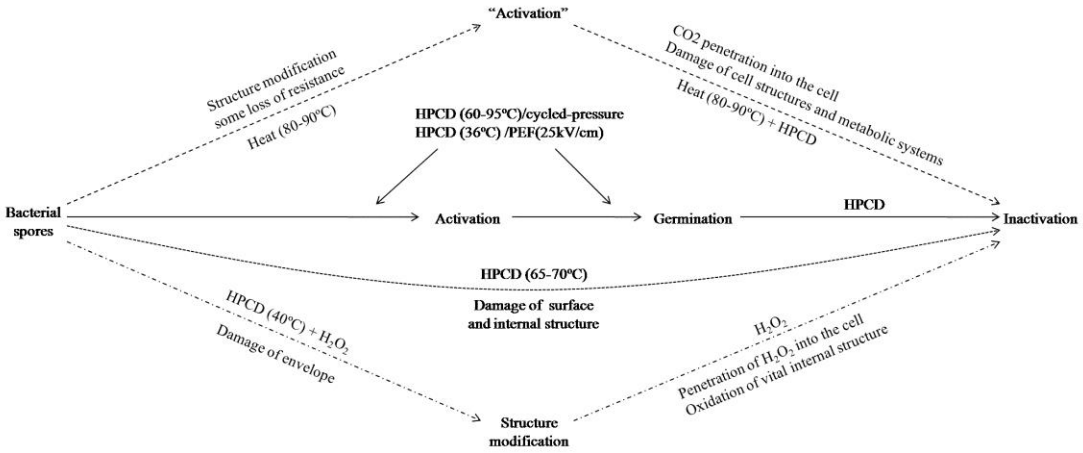

Figure 5 Inactivation of spores by different high-pressure CO_2 treatments. ‘ ’: inactivation of spores by high-pressure CO_2 (7-30 MPa, 60-95ºC, 2-6 h) (Watanabe et al., 2003b; Spilimbergo et al., 2003a), cycled-pressure high-pressure CO_2 (15 MPa, ΔP = 8 MPa, 30 cycles/h, 36ºC, 30 min) (Spilimbergo et al., 2002), and high-pressure CO_2 (30 MPa, 40ºC, 24 h) and PEF (25 kV/cm and 20 pulses) (Spilimbergo et al., 2003b). In these cases, the spores are first activated and germinated with a loss of the most resistance. The germinated spores become vulnerable to the bactericidal effect of CO_2 and are thus inactivated by high-pressure CO_2 treatment. However, the mechanism of spore activation and germination induced by high-pressure CO_2 is not clear; ‘ ’: inactivation of spores by high-pressure CO_2 (5 MPa, 80-90ºC, 60 min) with elevated temperature (Ballestra and Cuq, 1998). In this case, the spores are first “activated” by heat (80-90ºC), which makes the spores sensitive to the metabolic disturbance induced by CO_2. Then, the spores are inactivated by high-pressure CO_2 treatment; ‘ ’: direct inactivation of spores by high-pressure CO_2 (8-10 MPa, 65-70ºC, 30-40 min) (Bae et al., 2009). In this case, the high-pressure CO_2 treatment causes damage to the spore surface and internal structure that results in the inactivation of the spores; ‘ ’: inactivation of spores by a combination of high-pressure CO_2 and strong

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oxidant (such as H_2O_2; 27.5 MPa, 40ºC, 4 h) treatments (Zhang et al., 2006a). In this case, the spore envelope (spore cortex, inner and outer coat, and exosporium) is damaged by the high-pressure CO_2 and H_2O_2, and this damage allows the penetration of H_2O_2 and the oxidation of some vital internal structures, which results in spore death.

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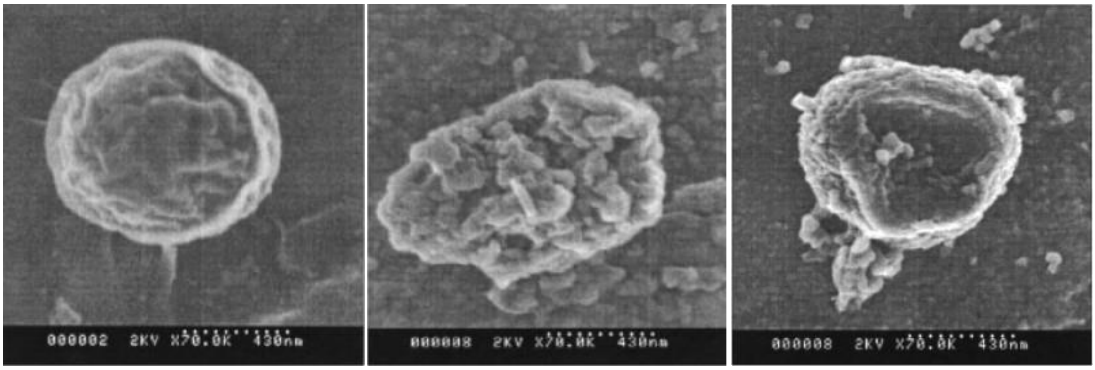

(a) (b) (c)

Figure 6 Scanning electron microscopy images of *B. cereus* spores: (a) untreated, (b) subjected to pulsed electric field treatment at 25 kV/cm and 20 pulses, (c) subjected to pulsed electric field treatment at 25 kV/cm and 20 pulses and the high-pressure CO_2 treatment at 20 MPa and 40ºC for 24 h (from Spilimbergo et al., 2003b).

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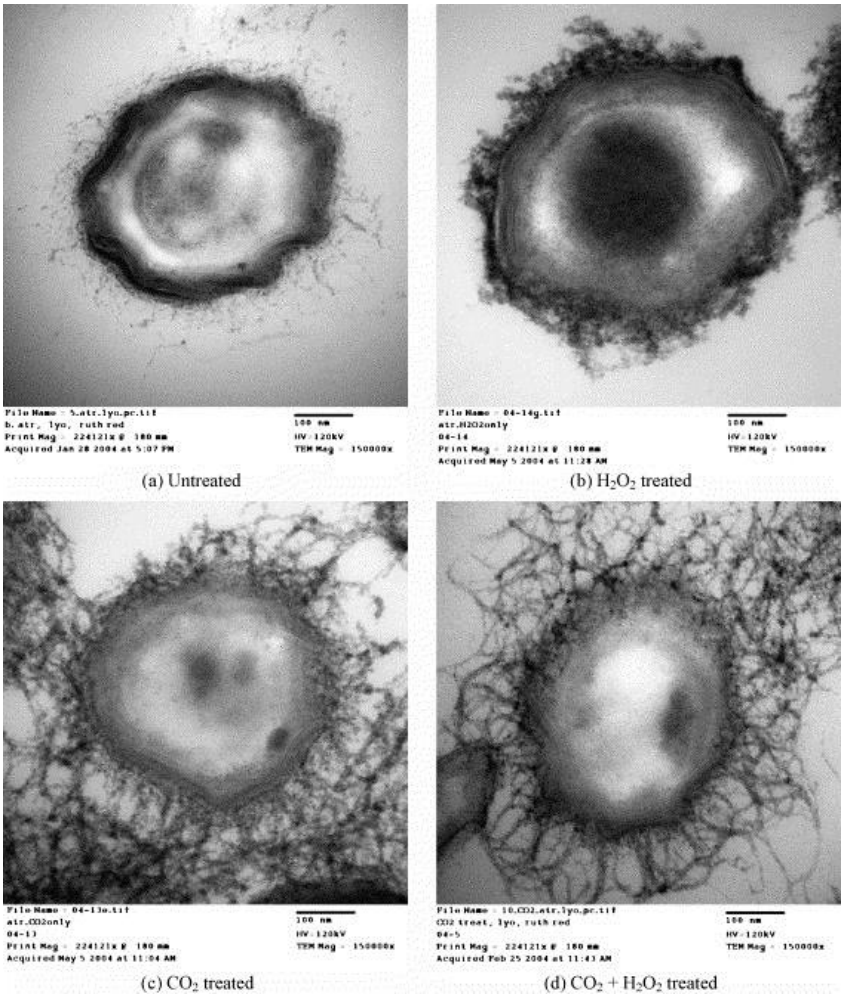

Figure 7 Transmission electron microscopy images of *Bacillus atrophaeus* spores (from Zhang et al., 2006b).

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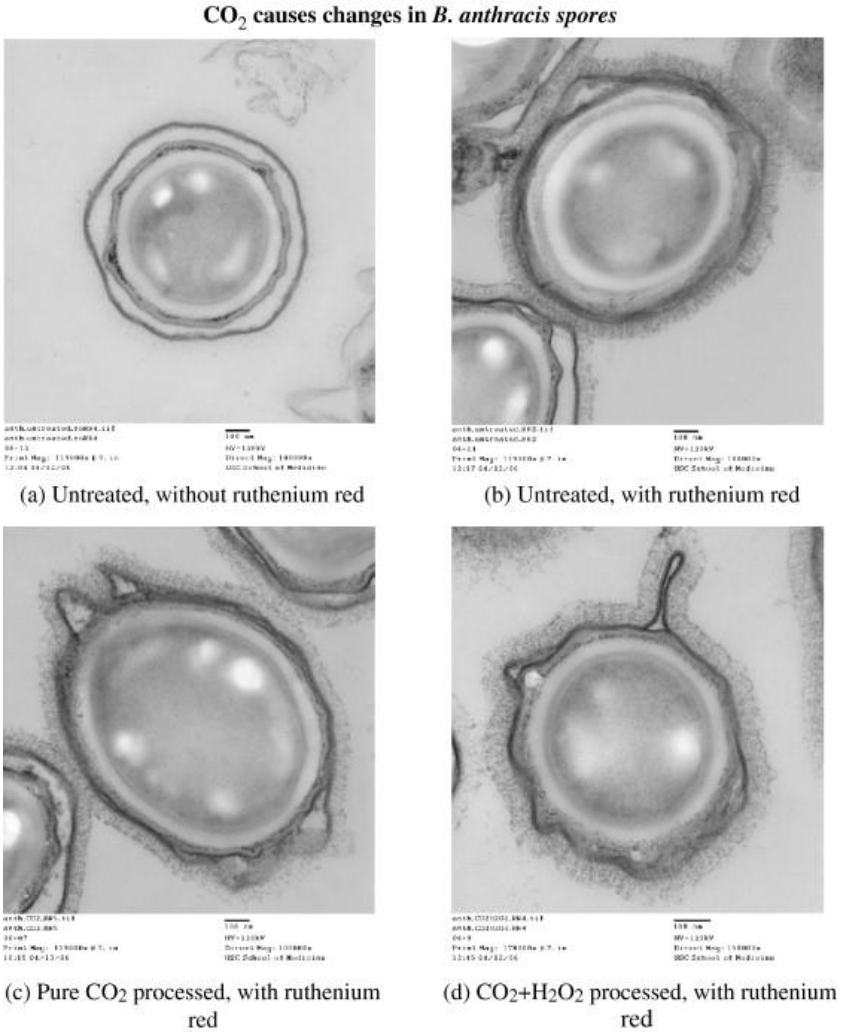

Figure 8 Transmission electron microscopy images of *Bacillus anthracis* spores (from Zhang et al., 2007).

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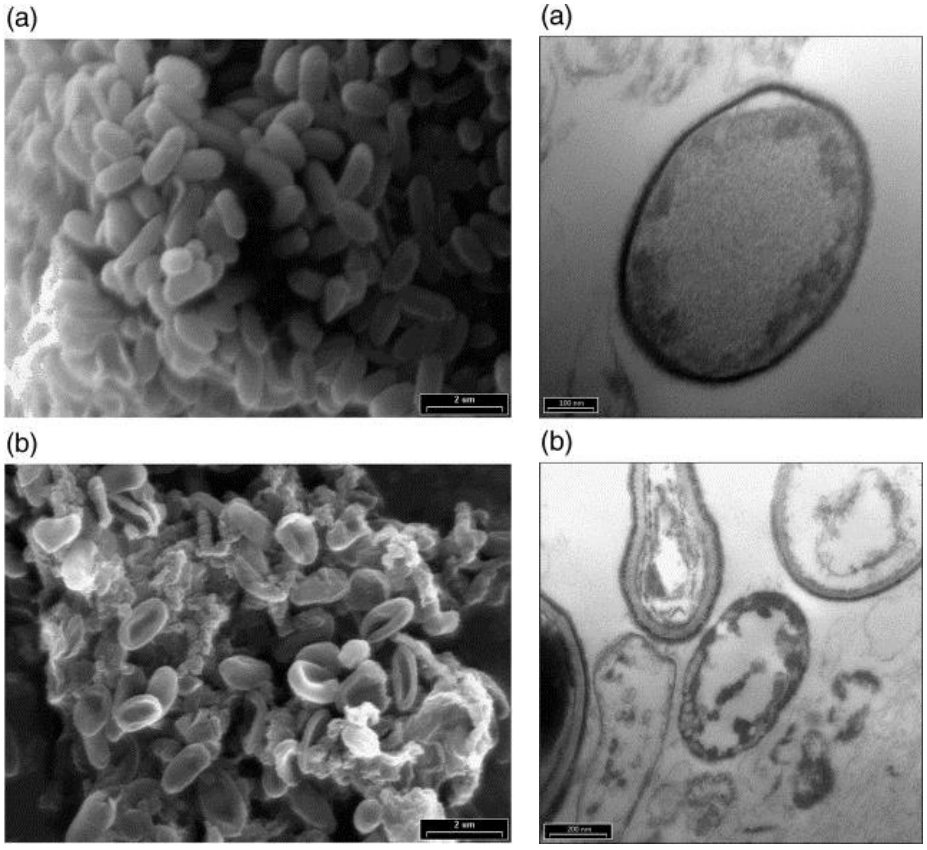

Figure 9 Scanning electron microscopy (right) and energy-filtering transmission electron microscopy (left) photographs of *Alicyclobacillus acidoterrestris* spores (a) before and (b) after high-pressure CO_2 treatment at 70ºC and 10 MPa for 30 min (from Bae et al., 2009).

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