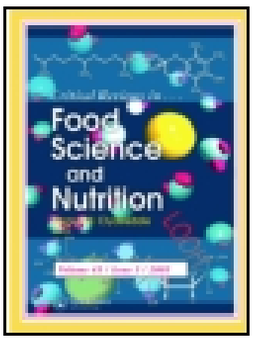

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Spore inactivation induced by cold plasma

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Abstract

Cold plasma has emerged as a non-thermal technology for microbial inactivation in the food industry over the last decade. Spore-forming microorganisms pose challenges for microbiological safety and for the prevention of food spoilage. Inactivation of spores induced by cold plasma has been reported by several studies. However, the exact mechanism of spore deactivation by cold plasma is poorly understood; therefore, it is difficult to control this process and to optimize cold plasma processing for efficient spore inactivation. In this review, we summarize the factors that affect the resistance of spores to cold plasma, including processing parameters, environmental elements, and spore properties. We then describe possible inactivation targets in spore cells (e.g., outer structure, DNA, and metabolic proteins) that associated with inactivation by cold plasma according to previous studies. Kinetic models of the sporicidal activity of cold plasma have also been described here. A better understanding of the interaction between spores and cold plasma is essential for the development and optimization of cold plasma technology in food the industry.

Keywords: Cold plasma; spore inactivation; factors; inactivation mechanisms; kinetic models.

INTRODUCTION

Spore-forming organisms exhibit resistances towards comrnon treatments (e.g., heat, cold, acid, bases, osmosis, desiccation) in the food industry (Shapiro et al., 1998). The survivals of spores in food products poses potential risks in terms of foodborne diseases or food spoilage. *Clostridium botulinum*, *Clostridium perfringens* and *Bacillus cereus* are prevalent foodborne-disease-related, spore-forming bacteria in food products (Wells-Bennik et al., 2016). *C. botulinum* can produce a type of neurotoxin and can lead to botulism-related foodborne diseases, which might cause muscle paralysis and even asphyxiation. *C. perfringens* is commonly associated with cooked meat products (Black et al., 2007). The foodborne diseases induced by *C. perfringens* are characterized by abdominal pains, diarrhea and vomit. *Bacillus cereus* can survive in a wide range of temperatures, pH values and water activities and frequently exist in protein-rich food products, such as meat. Heat labile toxins produced by *B. cereus* are usually associated with foodborne diseases, which are usually self-limiting and might have symptoms such as abdominal pain, diarrhea and nausea (Borge et al., 2001; Tewariand Abdullah, 2015). Once environment becomes

suitable, some endospores will germinate and produce metabolites, contributing to the occurrence of food spoilage. *Alicyclobacillus acidoterrestris* is a non-pathogenic, spore-forming, thermoacidophilic bacterium. However, the existence of *A. acidoterrestris* has been reported to be associated with the spoilage and change in favor of commercial pasteurized fruit juices, such as apple and orange juices (Silvaand Gibbs, 2001). Furthermore, *B. cereus* is not only associated with outbreaks of foodborne diseases but also causes spoilage of pasteurized dairy products, reducing shelf-life and resulting in substantial economic losses (Scheldeman et al., 2006). Therefore, efficient control of spores in food is a matter of great urgency for the food industry.

Conventionally, heat treatments has been the dominant methods for spore deactivation in food products (Byrer et al., 2000). Due to extreme resistance of spores, higher temperatures, such as ultra-high temperatures (UHTs), are required for efficient inactivation of spores (Melly et al., 2002; Setlow, 2007). However, harsh heat treatment is likely to have adverse effects on the freshness, nutrition and quality of food. Subsequently, high pressure (HP) has been proposed as an alternative technology to thermal treatment for inactivation of spores (Reineke et al., 2013). It is widely accepted that the sporicidal behavior of HP contains two steps including first germination-induction and final inactivation. Nevertheless, not all spores can be induced to germinate by HP treatment (Borch-Pedersen et al., 2017). In recent years, other alternative non-thermal technologies, including UV radiation, high pressure CO_2

(HPCD), electrolyzed water (EW), and cold plasma, have also been developed for spore inactivation in food products (Douki et al., 2005; Hertwig et al., 2015; Okull et al., 2006; Rao et al., 2016).

Cold plasma, also called non-thermal plasma, is emerging as a promising sterilization technology in the food industry (Liao et al., 2017). Plasma is produced by ionized or partially ionized gases, which consists of atoms, electrons, charged particles, radicals, ultraviolet (UV) photons, etc. In cold plasma, the temperature of electrons is much higher than the temperature of other particles, maintaining the plasma in a non-thermodynamic equilibrium state. Therefore, the overall temperature of cold plasma stays relatively low (<60℃) (Misra et al., 2016). Generally, the abundant reactive species in cold plasma are thought to be responsible for the efficient inactivation of microorganisms. Previous studies have proposed the application of cold plasma for inactivation of highly resistant spores (Butscher et al., 2015; Deng et al., 2007). Deng et al. (2006) found that a 10-min helium cold plasma treatment resulted in more than 99.99% reduction in *Bacillus subtilis* spores. However, there have been limited efforts given to elucidate how spores are inactivated by cold plasma. The exact mechanisms underlying sporicidal activity of cold plasma still remain unclear. More recently, cold plasma has been employed to inactivate spores in the food products, especially those with low water activity, such as wheat grains and red pepper powders (Table 1). For industrial applications of cold

plasma in the food industry, an adequate understanding of the sporicidal activity of cold plasma is needed.

In this review, we discuss the extrinsic and intrinsic factors that affect the resistance of spores to cold plasma. Based on previous studies, an overview of the mechanisms underlying spore inactivation by cold plasma is given. Finally, the kinetics of spore inactivation by cold plasma are also described in this review.

FACTORS AFFECTING THE RESISTANCE OF SPORES TOWARDS COLD PLASMA

Processing parameters

Processing parameters, such as gas types, treatment times, input powers and exposure modes, are important elements affecting the sporicidal capacity of cold plasma (Table 2). One of these parameters could alter the chemistry of the entire plasma, including the electron density, concentrations of charged and radicals, and amount of emitted UV photons. To date, the effect of gas types on spore deactivation by cold plasma has been most widely assessed by researchers (Hertwig et al., 2015; Song et al., 2012a; 2012b). Hertwig et al. (2015) compared the effect of pure argon, argon with 0.135% (v/v) oxygen, and argon with 0.135% (v/v) oxygen and 0.2% (v/v)

nitrogen on the resistance of *B. subtilis* spores to cold plasma. The sporicidal activity was highest for Ar with 0.135% (v/v) O_2 and 0.2% (v/v) N_2, which emitted UV light with the highest intensity. Though pure Ar-plasma had the lowest number of UV photons, treatments with this plasma resulted in greater reduction in spores count than Ar with 0.135% (v/v) O_2, which was attributed to the higher levels of reactive oxygen species and reactive nitrogen species (ROS/RNS) in pure-Ar plasma than those in Ar with 0.135% (v/v) O_2. Furthermore, the addition of a small amount of oxygen to helium plasma has been observed to increase the sensitivity of spores to cold plasma (Song et al., 2012a). Song et al. (2012b) found that the greatest reduction in *Candida albicans* spore count was achieved by using a gas mixture containing 99% He and 1% O_2, in comparison to helium gas with higher levels (4% and 7%) of O_2. Oxygen added to helium gas will collide with electrons, helping to provide higher levels of oxygen-related radicals, which then react with and damage spores. However, the electronegative property of O_2 allows it to easily absorb the electrons produced in plasma, leading to decreased electron energy and density. Therefore, higher O_2 concentrations might weaken the sporicidal activity of cold plasma. In sum up, a reduction level in spores by cold plasma is highly dependent on gas compositions. Exposure mode is another element that affects the resistance of spores to cold plasma (Dobrynin et al., 2010; Patil et al., 2014). Patil et al. (2014) observed that direct air cold plasma treatment for 60 s resulted in a more than 6-log reduction in *Bacillus atrophaeus*, while indirect treatment for the same duration led to inactivation

level by only 2.1 logs. The limited contact between spores and generated reactive species might inhibit the ability of cold plasma to kill spores. Generally, input powers (voltages or frequencies) and treatment times are directly proportional to the inactivation level of spores treated with cold plasma (Bussiahn et al., 2012; Butscher et al., 2015; Deng et al., 2006). Deng et al. (2006) proposed that enhancement of applied voltages increased the electron energy and density, contributing to increased radical species in plasma, which in turn react with spores. On the other hand, other parameters (e.g., flow rates) of cold plasma processing should be optimized to achieve maximum spore inactivation (Ouf et al., 2015).

Environmental factors

Common environmental elements such as matrixes (e.g., constitution and size), humidity (or water activity), or organic matters strongly influence the lethal effects of cold plasma on spores (Table 3). Hertwig et al. (2015) used cold plasma to treat *B. subtilis* endospores located on various surfaces, including glass petri dishes, glass beads and whole black peppercorns. They found that the highest reduction level was achieved with glass beads, while the lowest was achieved with peppercorns. This observation might be explained by the fact that *B. subtilis* spores were distributed more homogenously on the glass bead surfaces than on the other surfaces. Nevertheless, the spores on petri dishes or peppercorns readily agglomerated,

blocking the penetration by UV photons and reactive species produced by cold plasma. However, in a study by Klaempfl et al. (2012), no difference was observed in the reduction level of *Geobacillus stearothermophilus* levels on the various matrixes of stainless steels, glass, and polymeric surface (PVC and PTFE) surfaces after cold plasma treatment. Furthermore, the sizes of surfaces seem to play a role in the resistance of spores to cold plasma exposure. Kim and Choi (2017) treated a large and small red peppers inoculated with *B. cereus* with cold plasma. The reduction level in spore levels in the large peppers was 1.4 logs spores/cm^2, which was higher than the 0.8 log spores/cm^2 observed for small peppers. The authors explained that the higher ratio of surface area led to the reactive species in the plasma being distributed over a larger area, reducing the interactions with spores. In addition to matrix, environmental humidity is also an important determinant of the lethality of cold plasma on spores. Jeon et al. (2014) found that the reduction in the level of *G. stearothermophilus* increased by approximately 3 logs as the humidity increased from 5.5 to 17.9 g/cm^3. Higher humidity increased the production of more reactive species (e.g., $OH^{\cdot}$ and $H^{\cdot}$) via the dissociation of water molecules by electrons in cold plasma. However, contradictory results were observed in a study by Muranyi et al. (2008), who observed that the reduction in the level of *B. subtilis* spores was decreased from 4.7 to 2.6 logs as the relative humidity increased from 0 to 80% with a 1-s cold plasma treatment. The addition of water content was thought to reduce the homogeneous production of plasma, resulting in a decrease in the sporicidal activity of cold plasma.

On the other hand, for *Aspergillus niger* spores, an optimum relative humidity of 70% was determined to achieve a maximum spore reduction of 1.2 logs when compared with the 0.7 and 0.8 logs achieved at relative humidities of 0 and 80%, respectively. Therefore, the exact effects of humidity on spore inactivation by cold plasma remain to be uncovered. Generally, the addition of organic matters is likely to increase the resistance to cold plasma due to the protective effect of organic matter on spores (Connor et al., 2017; Klaempfl et al., 2014). Klaempfl et al. (2014) found there remained viable *Clostridium difficile* spores with additional burdens of 0.03% bovine serum albumin (BSA) even after 10 min of cold plasma treatment, while 5 min was enough for completed inactivation of spores in the absence of BSA.

Spore properties

The resistances to cold plasma have been observed to vary among various spore strains (Table 4). In a study by Connor et al. (2017), five genetic clades of *C. difficile* (ribotype 002, ribotype 027, ribotype 023, ribotype 017, ribotype 078) were subjected to He/O_2 cold plasma treatment. Spores of ribotypes 027 and 078 of *C. difficile* were found to be the most and least resistant to cold plasma exposure, respectively. The authors attributed this variation to the differences in spore coat structures among various strains. Similar spore strain-dependent spore inactivation behavior was observed for other disinfectant treatments, such as heat treatment (Berendsen et al.,

2015, Orsburn et al., 2008). Dobrynin et al. (2010) attributed the various degrees of cold plasma resistances of *B. cereus* and *Bacillus anthracis* spores to variations in the levels of virulence-related plasmids. However, in a study of Van Bokhorst-van De Veen et al. (2015), N_2-based cold plasma was found to have comparable inactivation effects on spores of non-heat-resisant *B. cereus*, heat-resistant *G. stearothermophilus*. The authors demonstrated that the determinants of heat resistance, such as the reduced water content in spore core and the ability to repair DNA damage, is less likely to associated with the resistance of spores to cold plasma. Therefore, data regarding the strain dependency of spore inactivation by cold plasma are inconclusive. Further efforts to reveal the effects of spore strains on the efficacy of inactivation by cold plasma are required to identify the reference strains for the assessments of cold-plasma-mediated pasteurization in practice.

INACTIVATION TARGETS OF SPORES FOR COLD PLASMA

Greater knowledge of the mechanism of inactivation of spores by cold plasma would assist in further optimization and development of cold plasma technology. To date, the exact mechanisms via which cold plasma inactivates spore cells remain unclear. Whether spore injury induced by cold plasma leads to germination remains unknown. Furthermore, the primary targets of cold plasma in spores have yet to be

determined. Based on previous studies, the proposed sporicidal modes of cold plasma are summarized in Figs. 1 and 2.

Outer structures

Generally, spores possess multilayered outer structures, as the first barrier to prevent the action of foreign chemicals or physical invasion; these outer structures are and as the main reason for the resistances of spores. Usually, spore outer structures, from the inside out, are composed of inner membranes, cortex, outer membranes and coats (Setlow, 2012). Some spore species, such as *B. cereus*, have an additional outermost layer, named the exosporium, which is made of proteins, lipids and carbohydrates. The main aim of spore coat layers is to inhibit the invasion by large molecules. Cortex layers are composed of peptidoglycans and play a significant role in spore resistance. Inner membranes, where fatty acids are abundant, are important for the marked impermeability of spore cores to small molecules. Some studies have proposed that the outer structures served as targets for spore inactivation by cold plasma (Deng et al., 2006; Raguse et al., 2016; Sun et al., 2012; Tseng et al., 2012; Van Bokhorst-van De Veen et al., 2015). In as early as in 2006, the completed rupture of *B. subtilis* induced by a helium atmospheric cold plasma were observed by scanning electron microscopy (SEM) (Fig. 3) (Deng et al., 2006). The author concluded that the oxidation by reactive plasma species was the main

contributor for the death of *B. subtilis*, while heat, UV photons and charged particles, electric field played a minor role. Tseng et al. (2012) demonstrated that spore coat damage induced by cold plasma led to leakage of dipicolinic acid (DPA) and increased the hydration of spore cores, which was a major cause of spore inactivation upon cold plasma treatment. Raguse et al. (2016) employed an atomic force microscopy (AFM) to observe the surface topography of plasma-treated *B. subtilis* spores and found the increased roughness and formation of cracks and fissures in the outer structures (Fig. 4). The authors also compared the resistances of wild-type *B. subtilis* spores and mutant spores lacking various coat layers (inner coats, outer coats, crust and encasement layers) to cold plasma. They observed that all the coat layers except for the crust part played important roles in protecting spores from cold plasma. Via direct imaging by phase contrast microscopy, Van Bokhorst-van De Veen et al. (2015) observed that N_2-based cold plasma treatment led to the phase changes of *B. cereus* spores from bright to grey because of water uptake, indicating the damages in outer structures (Fig. 5). Direct attack of spore coat layers by radicals from cold plasma disrupted the outer structures of spores, leading to the loss of integrity followed by leakage of cytoplasm and final spore death. However, the exact primary targets (components) of cold plasma on spore coat layers still remain unclear, and the elucidation of these targets requires further investigations to elucidate. In addition to the effect of radicals, ion bombardment or accumulation also results in destruction of the outer structure of spores through breaking bonds in the spore coats

(Opretzka et al., 2007). However, Raguse et al. (2016) reported that the influence of charged particles is minor to inactivate spores. The samples were in the center of plasma discharge, therefore, the voltage drop in front of samples was floating potential, which led to quite low ion energy. The contribution of charged particles in cold plasma to the damages in outer structures of spores still requires more investigations to elucidate.

DNA

Several studies have demonstrated that DNA in spores is the primary target of UV photons produced from cold plasma (Hertwig et al., 2017; Roth et al., 2012). The photoproducts from UV radiation of DNA in spores are thyminyl-thymine adducts, which are potential lethally to spores (Setlow, 2014). DNA plays significant role in carrying all the genetic information required for cellular metabolism. Once intracellular DNA is damaged, the spores are more likely to be dead. Roth et al. (2010) found that *B. subtilis* spores deficient in the nucleotide excision repair (NER) pathway and in α/β-type DNA-binding small acid-soluble proteins (SASPs) exhibited increased sensitivity to cold plasma, while spores lacking the spore photoproduct lyase (SPL) DNA repair pathway exhibited low sensitivity. Therefore, cold plasma induced DNA damage through mechanisms other than the formation of spore photoproduct. Elucidation of the exact mechanisms underlying cold plasma induced DNA damage in

spores requires further investigations. Hertwig et al. (2017) also observed that the α/β-type SASP-deficient mutant of *B. subtilis* exhibited increased sensitivity to cold plasma exposure, especially N_2-plasma, which produces high levels of UV photons. This observation indicates that UV radiation plays a dominant role in cold plasma induced DNA damage in spores. However, Muranyi et al. (2010) found that a 7-s cold plasma treatment resulted in an approximately 6-log reduction in the number of *B. atrophaeus* spores, while the DNA concentration decreased by approximately 1 log. Similarly, Tseng et al. (2012) observed no degradation of DNA in *B. subtilis* spores after a 20-min helium cold plasma treatment, while naked spore DNA was found to be severely fragmented. In a study of Fiebrandt et al. (2017), the authors found that the coat layers of spores did well in absorbing UV radiation, which shielded the DNA in the spore core from UV-radiation-based damage. This observation might be explained by the existence of abundant proteins in coat layers, which acted as the primary targets for photooxidation due to the present endogenous chromophores , such as amino acid side chains and bound prosthetic groups. Therefore, DNA in spores is not likely to be the primary target of cold plasma.

Metabolic Proteins

The critical metabolic proteins in spores include germinant receptors (GRs) (e.g., Ger A, Ger B, and Ger K), ion and DPA channels (e.g., SpoVA), and other

germinant-related enzymes, such as cortex lytic enzymes (CLEs) (e.g., CwlJ and SleB). Some studies have proposed that the key proteins could be adversely affected by cold plasma, resulting in the deactivation of spores (Dobrynin et al., 2010; Hertwig et al., 2017; Tseng et al., 2012; Wang et al., 2016). In a study by Laroussi et al. (2006), a distinct difference was observed in the use of carbon-based substances by untreated and cold-plasma-treated *Bacillus globigii* spores, which indicated that cold plasma might have effects associated with metabolic enzyme activities. However, the exact reasons for cold-plasma-induced heterogeneity in metabolic pathways of spores remains unclear. With the use of Raman spectroscopy and phase-contrast microscopy, Wang et al. (2016) compared the germination kinetics of untreated and cold-plasma-treated *B. subtilis* spores in the presence of the nutrient germinant L-valine and non-nutrient agents Ca^{2+}DPA and dodecylamine, which are activators of GerA, CLEs and SpoVAC proteins respectively, in spores. They observed that GerA proteins was the most vulnerable target of cold plasma treatment, since the L-valine-induced germination of spores was inhibited severely. On the other hand, cold plasma exposure resulted in minimal impacts on CLE proteins and least damages to the SpoVA proteins. Hertwig et al. (2017) applied cold plasma to treat a mutant *B. subtilis* lacking the key germination enzyme SleB, which is used for the production of DPA during sporulation. This mutant exhibited higher sensitivity to cold plasma than the wild-type strain. Lowering of DPA levels results in increased water content in the cores of the spores, which might affect the binding of α/β-type SASPs to

DNA and decrease the resistance of the cells to cold plasma exposure. The SleB-deficient mutant spores were more vulnerable to O_2 plasma than to air and CO_2 plasma, indicating that the germination-associated enzyme might be the target of ROS produced in plasma. However, the types of proteins that are more susceptible to cold plasma exposure than others remain to be identified in the future.

INACTIVATION KINETICS OF SPORES TREATED WITH COLD PLASMA

Reliable kinetic models are required for accurate description and prediction of inactivation rates by technologies used in the food industry. To date, many studies have established various types of inactivation models for the sporicidal activity of cold plasma. However, most of these models are primary inactivation models and fail to take both extrinsic and intrinsic elements, especially the physiological states of spores, into consideration.

Linear models

Some studies have indicated that the sporicidal activity of cold plasma follows first-order kinetic models, which are commonly used for thermal pasteurization of spores (Becker et al., 2005; Tseng et al., 2012). The linear models have the following form:

$$\mathrm{Log}_{10}\left(\frac{N_t}{N_0}\right) = -D \cdot t \quad (1)$$

In Eq. (1), N_0 is the initial spore concentration; N_t is the spore survivals at an exposure time of t; and D (decimal time) is the time required for one logarithmic unit reduction in spore concentration by cold plasma.

Liang et al. (2012) compared the first-order kinetic models, Weibull models and Simplified Baranyi models to fit the inactivation of *Penicillium expansum* spores by cold plasma. The coefficient of determination indicated a better fit with the first-order kinetic model in this case. Tseng et al. (2012) also observed that the survivals of *B. subtilis* spores during cold plasma treatment exhibited linear dependence on treatment times.

Nonlinear models

A biphasic inactivation model has been commonly used by most studies to describe the spore inactivation by cold plasma, in the following equation (Deng et al., 2006; Hertwigand Steins et al., 2015; Hury et al., 1998):

$$\frac{N_t}{N_0} = f \cdot e^{-k_1 \cdot t} + (1-f)e^{k_2 \cdot t} \qquad (2)$$

In Eq. (2), N_0 is the initial spore concentration; N_t is the population at an exposure time of t; f is a constant associated with the change from the first phase to the second phase; and k_1 and k_2 are the inactivation rates for the first and second phases, respectively.

Roth et al. (2010) employed a biphasic model to describe the inactivation of *B. subtilis* spores by cold plasma. The obtained inactivation rates for the first phase was 1.4 s^{-1}, which was much faster than the rate of 0.1 s^{-1} obtained for the second phase. Other studies have also found that the inactivation kinetics of spores exposed to cold plasma consist of one rapid linear phase followed by a slow phase. During the first phase, the spores on the surfaces are rapidly inactivated rapidly inactivated by cold plasma. Due to the limited penetration by reactive species, deactivate of shielded spores occurs during the second phase, which takes more time than the first phase.

Some studies applied the Weibull models to describe the sporicidal activity of cold plasma by using the following equation (Hertwig et al., 2015, 2017):

$$Log_{10}\frac{N_t}{N_0} = -b \cdot t^n \quad (2)$$

In Eq. (2), N_0 is the initial spore concentration; N_t is the concentration of surviving spores at an exposure time of t; b is the scale parameter and is thought to be linearly dependent on temperature; and n is the shape parameter, which is associated with the shapes of survival curves and the physiological effects of cold plasma exposure on surviving spores. If the value of n is lager than 1, it indicates that increased susceptibility of the remaining spores to cold plasma, as demonstrated by a survival curve with downward concavity. In contrast, an n value lower than 1demonstrates increased resistances of the remaining spores to cold plasma exposure. If n is equal to 1, each spore cell is equally susceptible to cold plasma.

Hertwig et al. (2017) fitted the inactivation data of *B. subtilis* to the Weibull models depending on the gas types used for generation of cold plasma. They found that the shape parameters for air, N_2, O_2 and CO_2 plasma were all less than 1, indicating that the remaining survivors of *B. subtilis* spores can adapt to applied cold plasma treatments and exhibited increased resistance to cold plasma.

In addition, the choices of kinetic models seem to depend on the types of cold plasma generated. Hertwig et al. (2015) found that the inactivation of *Bacillus* spores by a radio frequency (RF) plasma jet fitted well to a biphasic model, while a Weibull model was better suited for a microwave-generated cold plasma.

CONCLUDING REMARKS

Cold plasma has emerged as a novel non-thermal technology in the food industry over the last decade. Some efforts have been made to investigate the inactivation of spores by cold plasma in the last decade. However, there remain many questions to be answered in the future.

Due to differences in the equipment used by various studies, it is quite difficult to compare all the affecting elements and make general conclusions. Therefore, standards for processing protocols of cold plasma to achieve efficient inactivation of spores should be established based on additional scientific data.

Cold plasma is likely to destroy spore cells directly, inhibiting instead of inducing germination. However, the biochemical mechanisms underlying spore inactivation by cold plasma remain poorly understood. Further scientific and comprehensive investigations are required to elucidate and identify the exact targets in spore cells that are most vulnerable to cold plasma, thus providing a complete understandings of the inactivation mechanisms.

Furthermore, it is necessary to identify indicator spore strains for cold plasma pasteurization. The establishment of predictive mathematical models associated with the key affecting factors (e.g., gas types, humidity and spore resistances) for accurate description of the sporicidal activity of cold plasma is important for industrial-scale applications.

To date, few studies have focused on the application of cold plasma combined with other methods to inactivate spores. Therefore, the hurdle technology of combining cold plasma with other pasteurization technologies for efficient spore inactivation and maximum retardation of food qualities should be considered in future research.

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Figure captions

Figure 1. The proposed mechanisms underlying spore inactivation by cold plasma. In the first phase, most spores on the topside are rapidly inactivated by UV photons, radicals or ions from cold plasma. As the plasma particles penetrate deeper, the components of spores, including outer structures, DNA, and proteins, are gradually damaged and etched by reactive species (e.g., RNS/ROS and ions), leading to spore death.

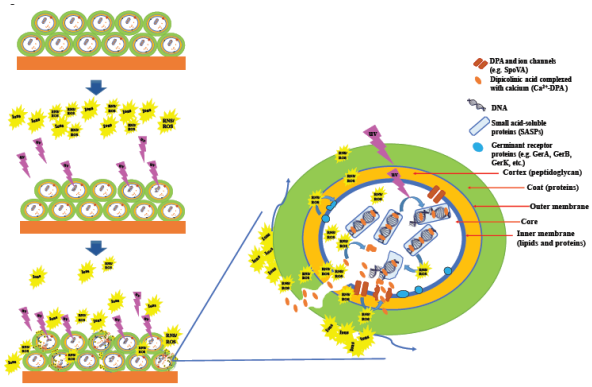

Figure 2. The possible targets (outer structure, DNA and metabolic proteins) in spores for cold plasma.

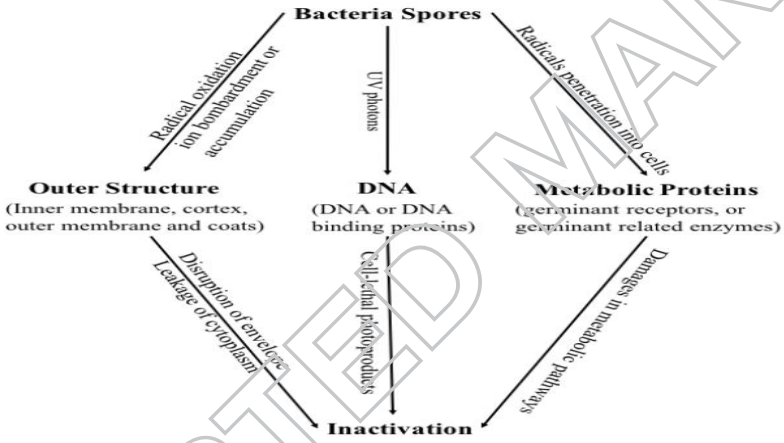

Figure 3. SEM photographs of the *Bacillus subtilis* spores (a) before and (b) after plasma treatment for 5 min with a ruptured spore point (Deng et al., 2006).

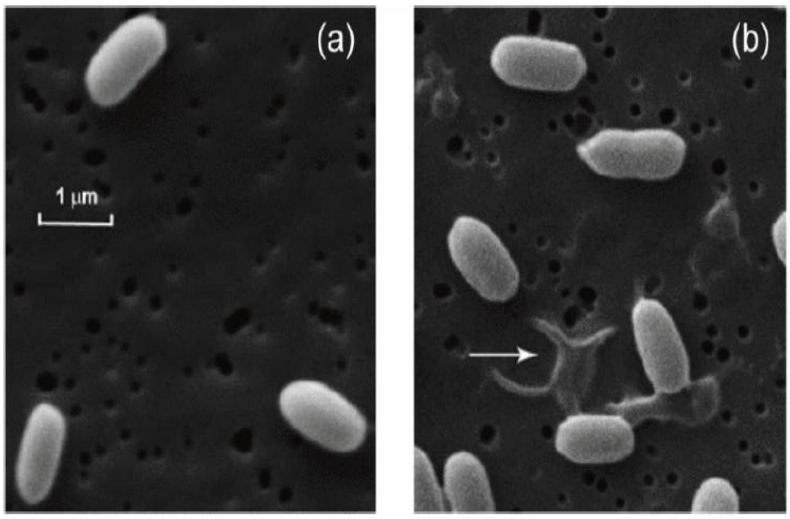

Figure 4. AFM images of plasma treated *B. subtilis* spores. Row (i)–(iii) represent height images, phase images, and 3D constructions, respectively. Untreated spores are as a control (a)–(c); argon plasma treated spores (d)–(f) for 15 s and (g)–(i) for 60 s; oxygen plasma treated spores (j)–(l) for 15 s and (m)–(o) for 60 s. Scale bars represent 500 nm (Raguse et al., 2016).

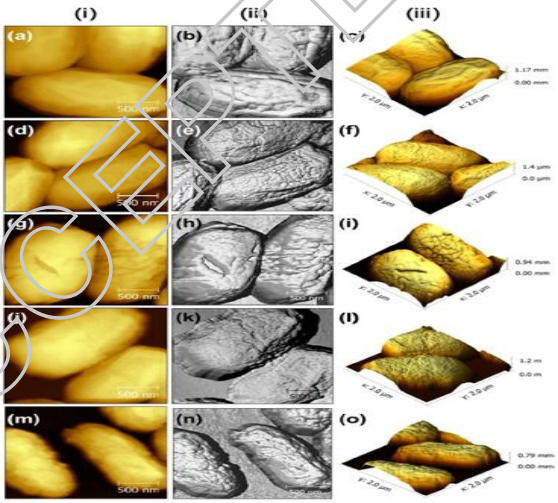

Figure 5. Phase contrast photographs of untreated (a) and plasma treated (b) spores after incubation for 0, 0.5 and 20 h without nutrients (Van Bokhorst-van De Veen et al., 2015).

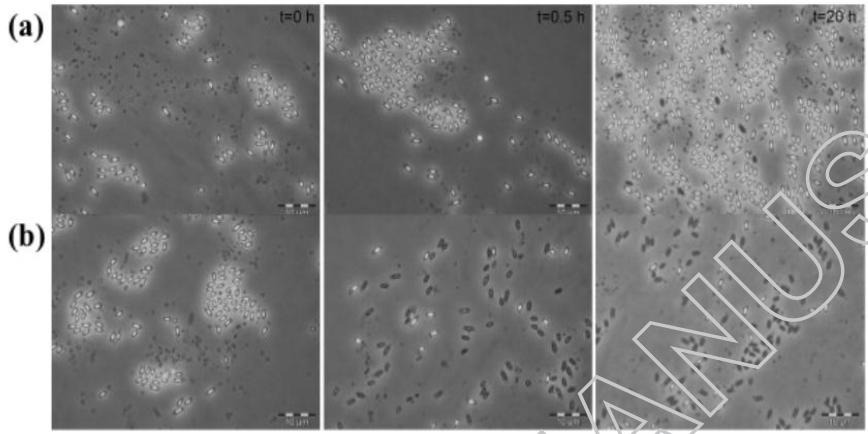

Table 1. The application of cold plasma for spore inactivation in food products

Food	Spores	Plasma types	Reduction	References
Red pepper	*Bacillus cereus*	Microwave power cold plasma	0.7~2.6 log spores/cm^2	(Kim et al., 2017)
Cereal	*Bacillus*	Air DBD	~5 logs	(Los et

grains	*atrophaeus*	plasma	spores/ml	al., 2017)
Onion powder	*B. cereus*	Microwave power cold plasma	1.1~2.1 log spores/cm^2	(Kimand Oh et al., 2017)
Pistachio	*Asphergillus flavus*	Air DBD plasma	>5 log CFU/sample	(Sohbatzadeh et al., 2016)
Date palm fruits	*A. flavus*	Double atmospheric pressure Argon cold plasma jet	980 CFU/100mm^2	(Ouf et al., 2015)
Milk powder	*Cronobacter sakazakii*	Microwave power cold plasma	0.9 log CFU/g	(Yeong et al., 2015)
Onion powder	*B. cereus*		0.4 log CFU/g	
Black pepper	*Bacillus subtilis, Bacillus atrophaeus*	Radio frequency plasma jet	2.4~2.8 log CFU/g	(Hertwig and Reineke et al., 2015)

Whole black peppercorns	*Bacillus subtilis*	Radio frequency (RF) plasma jet	Less than 1.0 $\log_{10}$	(Hertwig et al., 2015)

Table 2. The effect of processing parameters on spore inactivation by cold plasma

Spores	Plasma type	Matrix	Processing parameters	Reduction	References
Geobacillus stearothermophilus	Flexible sheet-type DBD	Stainless steel disk	Gas types (N_2 100%, N_2 80% + O_2 20%, N_2 50% + O_2 50%)	Higher reduction with higher O_2 level	(Etto et al., 2008)
Candida albicans	Atmospheric pressure glow discharge plasma	Glass plates	Gas types (He 100%, He 99% + O_2	He + 1% O_2 with highest reduction	(Song et al., 2012)

			1%, He 96% + O_2 4%, He 93% + O_2 7%)		
C. albicans	Atmospheric cold plasma brush	Qu artz plate	Gas types (He 100%, He 99% + O_2 1%, He 95% + O_2 5%, He 93% + O_2 7%)	He + 5% O_2 with highest reduction	(S ong et al., 2012)
B. subtilis	DBD	Pol ystyrene Petri dishes	Gas types (N_2, N_2+40ppm N_2O, N_2+220ppm N_2O,	N_2+40pp mN$_2$O> N_2+220ppmN$_2$ O> N_2+1000ppm N_2O>N_2,	(B oudam et al., 2006)

			N_2+1000pp mN$_2$O)		
B. subtilis (wild-type) and *B. atrophaeus*	Radio-frequency (RF) plasma jet	Glass petri-dishes	Gas types (Ar, Ar + 0.135%O_2, Ar + 0.135%O_2 + 0.2%N_2)	Ar > Ar + 0.135%O_2 + 0.2%N_2 > Ar + 0.135%O_2,	(Reineke et al., 2015)
B. subtilis (wild-type)	Radio frequency (RF) plasma jet	Glass petri-dishes, peppercorns, glass beads	Gas types (Ar, Ar + 0.135%O_2, Ar + 0.135%O_2 + 0.2%N_2)	Ar > Ar + 0.135%O_2 + 0.2%N_2 > Ar + 0.135%O_2,	(Hertwig et al., 2015)
B. subtilis (wild-type)	Diffuse coplanar surface barrier discharge	Glass beads	Gas types (Air, N_2, O_2,	N_2 > CO_2 > O_2>Air	(Hertwig et al.,

	(DCSBD) plasma		CO_2)		2017)
Clostridium difficile	Dielectric barrier discharge plasma jet	Microtitre plate wells	Gas types (He+ 0, 0.25, 0.5, 0.75 and 1% O_2)	He + 0.5% O_2 with highest reduction	(Connor et al., 2017)
B. subtilis	Radio-frequency (RF) polarization plate plasma	Glass slides	Gas types(O_2, N_2, Ar, vacuum)	O_2 > Ar > N_2 > vacuum	(Bengen Belgacem et al., 2017)
Aspergillus flavus	Microwave plasma	Red pepper powder	Gas types (N_2, He, N_2 + O_2, He + O_2)	N_2 > He > N_2 + O_2 > He + O_2	(Kim et al., 2014)
Bacillus atrophaeus	Atmospheric low temperature	Petri dish	Gas types (Air, 90% N_2	65% O_2 + 30% CO_2 + 5% N_2 >90%	(Patil et al.,

	plasma		+10% O_2, 65% O_2 + 30% CO_2 + 5% N_2)	N_2 +10% O_2 > Air	2014)
			Direct and indirect exposure modes	Direct > Indirect	
Bacillus amyloliquefaciens (BA) endospores	Low pressure plasma circulating fluidized bed reactor (PCFBR)	Wheat grain	Input powers (700 and 900 W)	900 W > 700 W	(Butscher et al., 2015)
B. subtilis	Atmospheric-pressure glow discharges	Membrane filters	Voltages (3.5, 4.65, 5.5, 6.5 kV)	Higher reduction with higher voltage	(Deng et al., 2006)
B. subtilis	Vacuum plasma	Glass slides	Treatment times	Higher reduction with longer	(Roth et al.,

				treatment time	2010)
Bacillus cereus	atmospheric-pressure dielectric-barrier-discharge (DBD) plasma	Distilled water and glass slides	Exposure modes	Indirect > direct	(Dobrynin et al., 2010)
A. niger	Atmospheric pressure argon plasma jet	Date palm fruits	Flow rate (2.5, 3.5, 4.5 L/min)	3.5 L/min with highest reduction	(Ouf et al., 2015)
B. cereus	Corona plasma	Plastic plates	Supply power types (direct current power and pulse power)	Pulse power > Direct current power	(Koval'Ova et al., 2013)

Table 3. The effect of environmental factors on spore inactivation by cold plasma

Spores	Plasma types	Environmental factors	Reduction	References
Bacillus subtilis	Radio frequency (RF) plasma jet	Matrix (Glass petri-dishes, peppercorns, glass beads)	Glass beads > Glass petri-dishes > Peppercorns	(Hertwig et al., 2015)
Geobacillus stearothermophilus	Low-temperature N_2 gas plasma	Matrix (Paper and stainless steels)	Paper > Stainless steels	(Kawamura et al., 2012)
Geobacillus stearothermophilus	Air surface microdischarge plasma	Matrix (Stainless steels, glass, PVC and	No difference	(Klaempfl et al., 2012)

		PTFE)		
Bacillus cereus	Microwave-integrated cold plasma	Matrix size (red pepper size of 1.5 × 1.5 cm and 0.5 × 0.5 cm)	Higher reduction with larger matrix size	(Kim et al., 2017)
		Water activity (a_w) (0.64, 0.72, 0.83, 0.93)	Higher reduction with higher a_w	
Clostridium difficile and *B. subtilis*	Surface micro-discharge air plasma	Organic matters (0.03% BSA)	Lower reduction with addition of organic matter	1(Klaempfl et al., 2014)
Clostridium	Dielectric barrier discharge	Organic matters	Lower reduction	(Connor et al.,

difficile	plasma jet	(0.03% BSA)	with addition of organic matter	2017)
		Dry and aqueous environment	Dry environment > aqueous environment	
Geobacillus stearothermophilus	Surface micro-discharge plasma	Absolute humidity (5.5, 10.4, 17.9 g m^{-3})	Higher reduction with higher absolute humidity level	(Jeon et al., 2014)
Bacillus atrophaeus	Atmospheric low temperature plasma	Relative humidity (0, 3, 10, 30, 50, 70%)	Higher reduction with higher relative humidity	(Patil et al., 2014)

				level	
B. subtilis	Cascade atmospheric DBD plasma	Relative humidity (0 - 80%)	Lower reduction with higher relative humidity		(Mura nyi et al., 2008)
Aspergillus flavus				Highes t reduction with 70% relative humidity	

Table 4. The effect of spore properties on spore inactivation by cold plasma

Spo res	Spore properties	Plasma type	Ma trix	Resistanc e	Ref erences

Clostridium difficile	Clades (TL178 (Clade 1, Ribotype 002); R20291 (Clade 2, Ribotype 027); CD305 (Clade 3, Ribotype 023); CF5 (Clade 4, Ribotype 017) and M120 (Clade 5, Ribotype 078))	Dielectric barrier discharge plasma jet	Microtitre plate wells	R20291 (Clade 2, Ribotype 027) with most resistance	(Connor et al., 2017)
Bacillus cereus, Bacillus atrophaeus, and *Geobacillus stearoth*	Strains	Nitrogen plasma jet	Petri dishes	*B. cereus* ≈ *B. atrophaeus*≈ *G. stearothermophilus*	(van Bokhorst-van De Veen et al., 2015)

ermophil us					
C. difficile, B. subtilis, G. stearoth ermophil us	Strains	Surface micro-discha rge air plasma	Dr y stainles s steel carriers	*B. subtilis > G. stearothermop hilus > C. difficile*	(Kla empfl et al., 2014)
B. cereus and *Aspergill us flavus*	Strains	Microwa ve plasma	Re d pepper powder	*B. cereus* spores > *A. flavus* spores	(Kim et al., 2014)
G. stearoth ermophil us, *B. subtilis*,	Strains	Surface microdischar ge (SMD) plasma	Sta inless steel	*G. stearother- mophilus* > *Bacillus pumilus* >	(Kla empfl et al., 2012)

Bacillus atrophaeus and *Bacillus pumilus*				*Bacillus atrophaeus* > *Bacillus subtilis*	
B. cereus and *Bacillus anthracis* (anthrax)	Strains	Atmospheric-pressure dielectric-barrier-discharge (DBD) plasma	Distilled water and glass slides	*Bacillus anthracis* (anthrax) > *Bacillus cereus*	(Dobrynin et al., 2010)
Clostridium botulinum Type A, *C. botulinum* Type E,	Strains	Low-temperature atmospheric plasma jet	Polystyrene microplate	*C. botulinum* Type A with highest resistance	(Tseng et al., 2012)

Clostridium sporogenes, *C. difficile*, *Clostridium perfringens*