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Endospore Inactivation by Emerging Technologies: A Review of Target Structures and Inactivation Mechanisms

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Abstract

Recent developments in preservation technologies allow for the delivery of food with nutritional value and superior taste. Of special interest are low-acid, shelf-stable foods in which the complete control or inactivation of bacterial endospores is the crucial step to ensure consumer safety. Relevant preservation methods can be classified into physicochemical or physical hurdles, and the latter can be subclassified into thermal and nonthermal processes. The underlying inactivation mechanisms for each of these physicochemical or physical processes impact different morphological or molecular structures essential for spore germination and integrity in the dormant state. This review provides an overview of distinct endospore defense mechanisms that affect emerging physical hurdles as well as which technologies address these mechanisms. The physical spore-inactivation technologies considered include thermal, dynamic, and isostatic high pressure and electromagnetic technologies, such as pulsed electric fields, UV light, cold atmospheric pressure plasma, and high- or low-energy electron beam.

INTRODUCTION

Humankind has faced the challenge of food preservation for thousands of years. One of the presumably oldest methods is preservation by heat, which includes simple methods, such as cooking and roasting, or combinations of thermal and chemical preservation methods, such as smoking. In addition to the formation of a unique flavor profile and the often-improved digestibility of cooked food, the application of moderate heat inactivates vegetative microorganisms, significantly enhancing the shelf life of heat-treated food.

However, some bacteria form highly resistant bacterial spores, which are the perfect vehicles for spoiling food or infecting humans, in response to stress conditions. An example of the remarkable resistance of spores is botulism, which is caused by the spores of *Clostridium botulinum*. Botulism was one of the main drivers in the establishment of the $12D_{121.1°C}$ inactivation concept for spores in canned food (Esty & Meyer 1922). The D-value or decimal reduction time (D_T) denotes the time necessary at a specific temperature to reduce the spore concentration to one-tenth of the original value. Hence, the 12D concept is based on intense over-processing of the treated food, as 10^{12} spores/g is the highest possible packing density of spores. Today, inactivation of 10^9 thermophilic spores per gram is an accepted sterilization process in dairy and beyond (Kessler 2002). During food processing with nonisothermal conditions, the dimensionless death value represents spore inactivation by the fully applied thermal process intensity (Kessler 2002). In addition to preservation by heat, many other physical preservation technologies have been developed, some of which target the same or different inactivation mechanisms.

A valuable method to ensure both consumer safety and high-quality minimally processed food is the concept of applying multiple-hurdle technologies (MHTs) (Leistner & Gorris 1995), which combines chemical, physical, or biological preservation methods as so-called hurdles. However, the use of physicochemical hurdles tends to decrease due to consumer demand for clean label and minimally processed food. Legal regulations have reduced the use of traditional hurdles, such as salt (Publ. Office Eur. Union 2012) and sugar (Norton et al. 2006), because of health-related issues associated with excessive consumption of these compounds (Chaudhary et al. 2018).

To face these challenges, a multitarget strategy for controlling or inactivating bacterial spores may represent a possible processing option to tackle different endospore-inactivation mechanisms in food. Emerging physical processes for potential bacterial spore inactivation are the focus of this review.

ENDOSPORE STRUCTURE

In general, two orders of bacterial spores are relevant for food spoilage, Bacillales (aerobic or facultative anaerobic) and Clostridiales (anaerobic). The mature endospore exhibits multilayered morphology (**Figure 1**) and has several defense mechanisms to withstand harsh environmental conditions and preservation technologies.

From the outside, the first morphological compartment is the spore exosporium, which is not synthesized by all spore-forming bacteria. The next layer is the coat, which is, at least for *Bacillus subtilis*, composed of the crust, the outer and inner coats, and the basement (Setlow 2012). In addition to small amounts of carbohydrates and lipids, the coat layers are composed of tyrosine- and cysteine-rich proteins. The appearance and thickness of the spore coat layers among different species can vary. For example, the coat appears to be thinner and more compact for *Bacillus anthracis* and some *Bacillus cereus* strains. These multiple layers represent the first line of defense for endospores, acting as a filter for different chemicals, such as oxidizing agents, aldehydes, bases, acids, and alkylating agents (Setlow 2006), and playing a minor role in resistance to wet heat and UV light. Presumably, the most crucial role of the coat with respect to spore resistance is

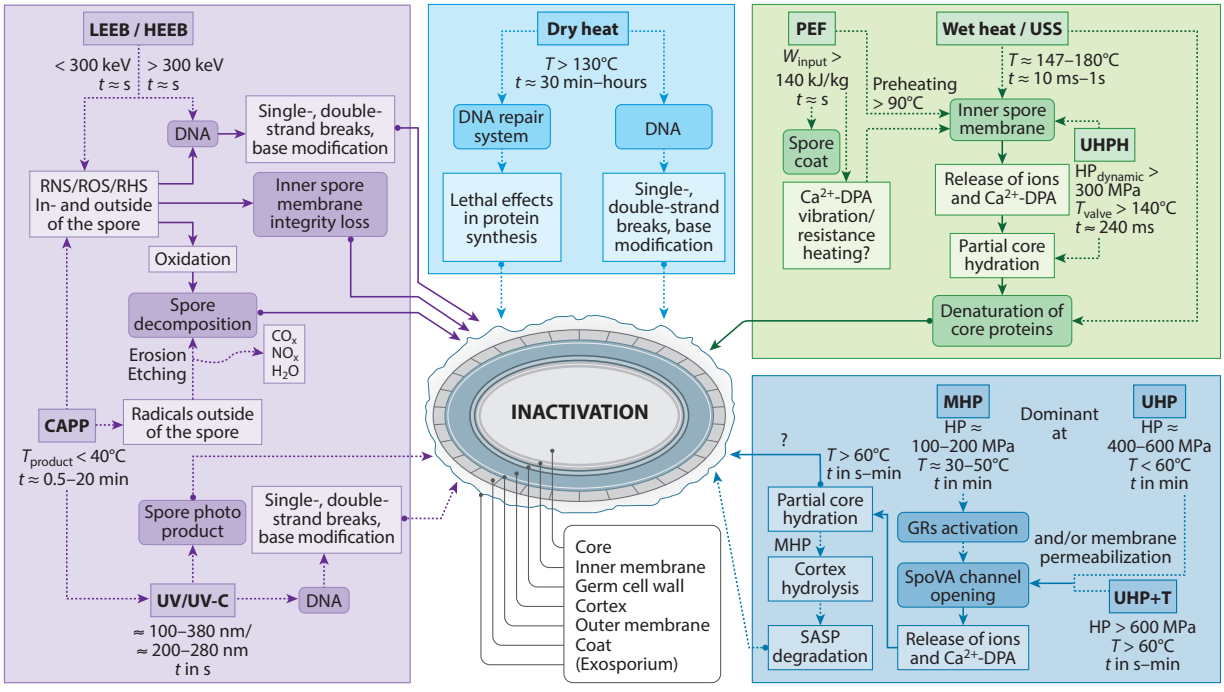

Figure 1

Overview of target structures and inactivation mechanisms observed in *Bacillus* and *Geobacillus* spores by different physical technologies: high pressure (HP), moderate high pressure (MHP), ultra-high pressure (UHP), ultra-high pressure combined with temperature (UHP+T), ultra-short sterilization (USS), ultra-high-pressure homogenization (UHPH), pulsed electrical fields (PEF), low-energy electron beam (LEEB), high-energy electron beam (HEEB), ultraviolet (UV) light, and cold atmospheric pressure plasma (CAPP). Solid arrows indicate joined pathways, and dashed arrows indicate individual pathways. Main target structures are shown in filled round shaped boxes. Abbreviations: DPA, dipicolinic acid; GRs, nutrient germination receptors; RHS, reactive hydrogen species; RNS, reactive nitrogen species; ROS, reactive oxygen species; SASP, small acid-soluble protein; spoVA, proteins that are encoded by the spoVA operon, which participates in channel formation in the inner membrane.

suppression of spontaneous germination (Jenkinson et al. 1981). Furthermore, for at least *B. subtilis*, one cortex lytic enzyme (CLE), CwlJ, is present on the coat layer and/or in the spore cortex (Setlow et al. 2017).

Adjacent to the outer spore membrane is the second major compartment of a spore, the peptidoglycan layer, which consists of the thick cortex and the germ cell wall (Setlow 2003). Whereas the peptidoglycan composition of the germ cell wall is similar to the composition of a vegetative cell, the peptidoglycans in spores are less cross-linked in the cortex (Wells-Bennik et al. 2016), which impacts spore defense against peptidoglycan active enzymes such as lysozyme. Related to spore resistance, the cortex has several unique characteristics and is crucial for core dehydration and spore dormancy (Dirks 1999). The basic molecular structure of these layers is highly conserved among different *Bacillus* and *Clostridium* species. The significant difference between these two genera lies in the level of the de-*N*-acetylation of glucosamine, which can affect the access of CLEs and, with regard to food preservation, also of the preservative lysozyme (E1105) to the peptidoglycan substrate (Atrih & Foster 2001). Another CLE, SleB (a more general designation for other spore strains is simply Sle), is found in *B. subtilis* and is present on the inner membrane's outer surface and in additional outer layers (Setlow et al. 2017). Sle and CwlJ orthologs can be found in various spore-forming bacteria, including *Clostridium* and *Bacillus* species (Henriques &

Moran 2007). However, *Clostridium* spores can have additional CLEs (Paredes-Sabja et al. 2011), and some, but not all, enzymes that cleave peptidoglycan have been identified. The cortex ensures the structural integrity of the spore while withstanding the turgor pressure generated by the high concentration of solutes in the core (Popham 2002). The third compartment, the core, is surrounded by the inner spore membrane, which is the analog of a cytoplasmic membrane of a vegetative cell. However, the volume of the spore core is smaller than would be expected based on the quantity of phospholipids, indicating a very dense structure of the inner membrane, which results in a strong permeability barrier. This membrane is in a gel-phase state (Cowan et al. 2004), and its water permeability is 3–4 orders of magnitude lower than prototypical lipid bilayer membranes (Sunde et al. 2009), resulting in a strong permeability barrier that protects the spore core from dehydration important for spore dormancy. Further embedded in the inner spore membrane are proteins responsible for the uptake and release of calcium dipicolinic acid (Ca^{2+}-DPA), such as spoVA proteins and the spore's nutrient germination receptors (GRs) (Li et al. 2012).

Despite having high levels of Ca^{2+}-DPA, the spore core also contains small acid-soluble proteins (SASPs), DNA, ribosomes, and enzymes, all of which are required for reactivation of metabolism and are supposed to be in an insoluble gel state (Sunde et al. 2009). In combination with the dense inner spore membrane, the high levels of divalent cations and Ca^{2+}-DPA are the primary factors responsible for core dehydration (Pedraza-Reyes et al. 2012). Ca^{2+}-DPA strongly contributes to the state of the core. Contributing 5–15% of the spore's dry weight (Popham et al. 2012), the total concentration of Ca^{2+}-DPA is well above the solubility level, indicating that it is in an insoluble form in the core. However, the individual levels of Ca^{2+}-DPA present in a spore are highly strain dependent (Lenz 2017, Reineke 2012) and can further differ for individual spores (Huang et al. 2007, Setlow et al. 2012). The core pH in dormant *Bacillus* spores is between 6.3 and 6.4 (Setlow & Setlow 1980), which, combined with the low water content, prevents protein unfolding, protonating, and deprotonating, consequently stabilizing the structure. Of special interest in this regard are the abovementioned SASPs. These small proteins are conserved across species and can bind to double-stranded DNA but not to RNA or single-stranded DNA, thus acting to protect the DNA (Setlow 2007). These defense mechanisms enable bacterial spores to survive not only most food preservation methods used but also extremely harsh conditions on earth or even in space (Horneck et al. 2012).

PHYSICAL INACTIVATION TECHNOLOGIES

To fulfill the demand for safe, natural, fresh-like, and minimally processed food, different thermal and nonthermal processes have been investigated, some of which not only exhibit lethal effects on vegetative microorganisms but also inactivate bacterial endospores. Only these technologies and their effects on target structures in the spore are discussed in the following sections.

Thermal Inactivation and Emerging Ultra-Short Sterilization

One of the most commonly applied methods of inactivating bacterial spores is the application of dry or wet heat. This method can significantly increase the shelf life of low-acid foods, leading to full inactivation of bacterial spores and resulting in a commercially sterile product (death value of 9 or 12 $\times$ $D_{121.1°C}$) (Kessler 2002). However, even though this technology has been used for more than a century to sterilize food and represents the most commonly used sterilization method, the inactivation mechanisms are still not fully understood, and further research in this area is needed to reduce the thermal load of thermally sterilized products and ensure consumer safety.

The highly dehydrated core immobilizes core proteins and hence prevents irreversible aggregation and/or denaturation (Chirakkal et al. 2002, Cowan et al. 2003, Sunde et al. 2009). In

combination with the high levels of divalent cations and SASPs surrounding the DNA, these are the primary factors for the high wet-heat resistance of spores (Setlow 2007). Wet heat can cause permeabilization of the inner spore membrane (**Figure 1**), for example, by denaturing membrane proteins (Coleman et al. 2007). Hence, core hydration accompanied by the release of Ca^{2+}-DPA (Janssen et al. 1958) occurs, resulting in protein mobilization and, consequently, reduced thermal resistance and possibly denaturation. If the proteins or enzymes required to reactivate the metabolism of the germinating spore are among them, the spore is inactivated. For *Bacillus megaterium* and *B. cereus* (Coleman et al. 2010), the inactivation of core enzymes is the rate-determining step for spore inactivation. Additionally, spores that lack SASPs are more sensitive to wet heat (Pedraza-Reyes et al. 2012), but wet heat does not cause DNA damage (Setlow 2006). In addition, the target proteins that are denatured have not yet been identified (Zhang et al. 2010). Contrary to inactivation by wet heat, dry heat does cause DNA strand breaks (Setlow 2007). Furthermore, Barraza-Salas et al. (2010) demonstrated that two DNA repair enzymes of *B. subtilis*, Nfo and ExoA, are affected by dry heat, leading to lethal defects in protein synthesis during and after germination (**Figure 1**). Hauck-Tiburski et al. (2019) also showed that the application of a specific combination of temperatures up to 150°C with moderate pressure up to 0.6 MPa retains liquid water in the spore core, resulting in higher *B. subtilis* spore inactivation (between 2.4 and 4.9 $\log_{10}$ at 150°C, 120 s, and an a_w of 0.5 in powder) compared to treatment at atmospheric pressure (0.7 $\log_{10}$), during which water rapidly evaporates because it has reached its boiling point. In addition to the impact on DNA, high heating rates (10^5°C/s) to temperatures greater than 200°C can cause structural damage, presumably because of ultra-fast water evaporation occurring inside the spore (Zhou et al. 2015).

An emerging technology for the preservation of liquid and other pumpable foods is ultrashort sterilization (USS). For this process, temperatures above the classical ultra-high-temperature (UHT) treatment of 135°C (up to 150°C) are used with treatment times in the millisecond range (Mathys 2018, Morgan et al. 1996). This fast heating is achieved using high surface-area-to-volume ratios (Georget et al. 2013), sometimes up to 100-fold higher than conventional equipment (Emig & Klemm 2005, Freund & Sundmacher 2011, Kiwi-Minsker & Renken 2005), or by direct innovative steam injection (ISI) (van Asselt et al. 2008). With regard to microorganism inactivation in general and for endospores in particular, published data are limited (Mathys 2008, 2018; Morgan et al. 1996; van Asselt et al. 2008; Zhou et al. 2015). However, the underlying concept for this innovative sterilization approach can be explained using the basic concept for the calculation of thermal inactivation. Here, the inactivation of an endospore or vegetative microorganism or a degradation reaction during food processing is quantified with a decadic logarithm of concentration changes under isothermal conditions:

$$\log_{10}\left(\frac{C(t)_T}{C_0}\right) = -\frac{k_T}{2.303}\ .t. \qquad 1.$$

The initial concentration of the compound of interest or microorganism is represented as C_0, and the concentration $C(t)$ is considered after treatment at a certain isothermal temperature level T. k_T is the reaction rate or velocity rate constant with holding times t. With this relationship, the thermal decimal reduction value D_T is calculated using the following equation:

$$D_T = \frac{2.303}{k_T}. \qquad 2.$$

Equation 2 represents the time needed to reduce the concentration of the compound of interest by 90%. The temperature dependence of the rate constant k_T can be calculated using the Arrhenius

equation:

$$\ln\left(\frac{k_T}{k_0}\right) = -\frac{E_a}{R}\cdot\frac{1}{T}. \qquad 3.$$

Here, k_0 is the velocity constant for $1/T = 0$, E_a is the energy of activation, and R is the universal gas constant. The thermal decimal reduction value D_T and the activation energy E_a are sufficient to characterize the thermal inactivation of a microorganism or a food compound (Emig & Klemm 2005, Kessler 2002). On the basis of these equations, the calculation of isorate lines for the inactivation of vegetative microorganisms, endospores, and unwanted degradation reactions is possible.

By using this concept and the limited existing data on ultra-short high-temperature treatments, processing windows for ultra-short pasteurization (USP) and USS can be defined (Mathys 2018). Regarding spore inactivation, the denaturation of spore core enzymes, α- and β-SASP denaturation, and other factors involved in spore inactivation by wet heat (**Figure 1**) presumably have a k_T, D_T, and E_a, as reported for many proteins (Kessler 2002).

Despite the impact of different temperature and time domains used for spore inactivation, the factors that contribute to spore heat resistance in detail are unknown. Berendsen et al. (2016) provide conclusive evidence that in *B. subtilis*, the spore heat resistance is due to the presence of a mobile genetic element (Tn1546-like) and, in particular, the spoVA^{2mob} operon. As these spoVA operons are conserved in different *Bacillus* species, it is assumed that they contribute at least to heat resistance in *B. subtilis*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* (Berendsen et al. 2016). Furthermore, the heat resistance of individual spores and spore populations has a tremendous impact on process reliability and, consequently, consumer safety and is affected by the environmental conditions experienced during sporulation. These conditions include intrinsic factors, such as nutrient composition, pH, and dry stress, and extrinsic factors, such as temperature, which is by far the more thoroughly investigated, as it exerts a direct impact on wet heat as well as the pressure resistance of the mature spore. A higher thermal resistance does correlate with higher sporulation temperature, as shown by several authors for *B. subtilis* (Palop et al. 1999), *B. licheniformis* (Baril et al. 2012), *Clostridium perfringens* (Paredes-Sabja et al. 2011), and *Clostridium botulinum* (Lenz & Vogel 2014). Heat stress during sporulation induces the synthesis of more than 60 proteins, including 11 heat-shock proteins (Lenz 2017). Some of these proteins are involved in repairing misfolded proteins and damaged cellular components (Movahedi & Waites 2000) and others in stabilizing the tertiary and quaternary structure of macromolecules (Khoury et al. 1990).

In contrast, *B. subtilis* under cold stress during sporulation induces the *des* gene coding for Δ5-lipid desaturase expression, which produces unsaturated fatty acids (Aguilar et al. 2001), thus changing the physical state of the membrane phospholipids and subsequently affecting the high-pressure (HP) resistance of a spore.

High Pressure

Every chemical or biochemical reaction proceeds under pressure. This could be in a vacuum (<0.1 MPa), at atmospheric conditions (0.1 MPa) or slightly above, as in an autoclave, or at HP, as is used in food preservation (>300 MPa). The effect HP exerts on organic macromolecules is often described using Le Châtelier's principle, which states that if a system at equilibrium experiences a change in temperature, concentration, and/or volume, the equilibrium shifts to establish a new equilibrium. Regarding HP, any phenomenon that is accompanied by decreased reaction volume is enhanced, resulting in changes in parameters such as pH, tertiary and quaternary structure of proteins, and steric structure of lipids (Georget et al. 2015, Mathys & Knorr 2009). Because of the microscopic ordering principle, an increase in pressure at constant temperature increases the

ordering of a substance and decreases the free volume of the system. The transition from state A to state B for an equilibrium process is described by Gibbs free energy (G), which is a general approach to denoting molecular or physicochemical states, depicting an elliptical transition line for $\Delta G = 0$ at various pressures and temperatures (Heremans 2002).

Isostatic high pressure. The fundamental physicochemical effects of HP have a direct impact on bacterial spores. Generally, pressures below 1 GPa have no impact on covalent bonds (Cheftel 1995). In a vegetative cell, the hydrogen bonds in the DNA helix are stabilized by HP (Winter & Dzwolak 2005), leading to the assumption that the DNA stabilized by SASPs in the dehydrated spore core is likely not affected by HP and, hence, is not the primary target structure for the HP-mediated inactivation of spores.

Contrary to the stabilizing effect of HP on DNA, lipid systems, such as membranes, are affected by pressure and can undergo phase transition or fluidity changes (Kato & Hayashi 1999, Winter & Dzwolak 2005). Membrane complexes seem to be the major target for HP-driven spore inactivation (Reineke et al. 2013b). The outer spore membrane is presumably affected by HP but is not essential for spore germination. In contrast, the inner spore membrane acts as the cell membrane of the germinating spore and ensures spore core dehydration. Hence, a pressure-induced phase transition or fluidity change can alter the permeability properties of the inner spore membrane, leading to release of Ca^{2+}-DPA during the pressure treatment (Reineke et al. 2013c). Subsequent core hydration is followed by higher mobility of core proteins with reduced heat resistance of the spore (**Figure 1**).

Consequently, the ability of a spore to retain Ca^{2+}-DPA under HP at a certain process temperature is a major factor for the pressure resistance of a single spore, spore strains, or spore species (Margosch et al. 2004, Reineke et al. 2013c). Although much research has been done on the behavior of biological membranes under pressure (Winter & Jeworrek 2009), data on the exact behavior of the inner spore membrane under pressure are scarce but support this inactivation mechanism (Georget et al. 2014a, Hofstetter et al. 2013). However, further research is needed to clarify the role of the inner spore membrane as the primary target for HP-driven spore inactivation, especially in combination with elevated process temperatures (>60°C) and for pathogenic *Clostridium* strains.

The effect of the sporulation temperature on the pressure resistance of *B. cereus* (Raso et al. 1998), *B. subtilis* (Igura et al. 2003, Melly et al. 2002), and *C. botulinum* (Lenz & Vogel 2014) showed increased HP resistance and increased heat sensitivity for lower sporulation temperatures. At lower sporulation temperatures, more polyunsaturated fatty acids are integrated into the inner spore membrane, altering physical properties of the spore by increasing membrane fluidity (Cortezzo & Setlow 2005). This finding indicates that alterations in the fatty acid composition of the inner spore membrane (Abee et al. 2011) are crucial for spore survival under pressure. Furthermore, Melly et al. (2002) found significant alterations in spore cortex and coat composition for *B. subtilis* at different sporulation temperatures. Modifications in the coat-associated proteins of *C. botulinum* type E spores in response to varying sporulation conditions (temperature and cation composition) that affect HP resistance were also reported by Lenz (2017). An effect of mineral content on resistance of *B. subtilis* to moderate HP (<300 MPa) was also reported by Igura et al. (2003), whereas pressure resistance increased after demineralization of the spore and decreased after remineralization with Mg^{2+} and Ca^{2+}, but not with K^+ or Mn^{2+}, suggesting that Mg^{2+} and Ca^{2+} might be involved in the activation of CLEs during non-nutrient spore germination.

Another class of biomolecules affected by HP is the proteins, and HP produces particular impacts on their quaternary and tertiary structures (Buckow & Heinz 2008, Winter & Dzwolak 2005) and, consequently, their biochemical function and ability to interact with membranes

and membrane proteins. Winter & Jeworrek (2009) reported that membrane proteins can be very pressure stable, but they can dissociate, weakening their interaction with membranes (Lenz 2017) and exerting a significant impact on endospore survival under pressure. The inner spore membrane consists of several membrane proteins, including pressure-sensitive spore Ca^{2+}-DPA channels (Paidhungat et al. 2002) and GRs (Setlow 2003), which are both essential for spore germination, underlining the important role of the inner spore membrane for not only pressure-induced inactivation but also non-nutrient spore germination.

Application of relatively low pressures (100–400 MPa) and nonlethal temperatures (<50°C) can trigger GRs of *Bacillus* (Mathys et al. 2007a, Reineke et al. 2012) and some *Clostridium* spores (Doona et al. 2016, Lenz 2017) to initiate spore germination (**Figure 1**). Following the HP trigger, Ca^{2+}-DPA is released, and, presumably, the spore cortex is at least partially degraded, even under pressure (Doona et al. 2016; Reineke et al. 2013b,c); this degradation is accompanied by a loss of spore resistance to moderate heat treatment (Borch-Pedersen et al. 2017, Lenz et al. 2015, Reineke et al. 2013c), which is a crucial step in the loss of resistance properties and demonstrates an interesting hurdle that could be utilized by a variety of sterilization approaches (**Figure 1**). Wuytack et al. (1998) and Reineke et al. (2013a) reported that *B. subtilis* spores are even able to degrade their SASPs, as they are sensitive to hydrogen peroxide treatment and showed no inner structure by sectioned micrographs.

The precise mechanism of GR activation in response to mild pressure is still unknown, but levels of expressed GRs seem to be a major determining factor (Doona et al. 2014). Furthermore, different types of GRs, such as GerA, GerB, and GerK, of a spore strain or different spore species or genera show distinct pressure sensitivities (Black et al. 2005, Borch-Pedersen et al. 2017) or do not respond to pressure, as shown in *Clostridium difficile* (Doona et al. 2016). This hypothesis is backed by Doona et al. (2016), who showed that *C. difficile* spores, which lack inner membrane GRs, exhibited no germination events in response to 150-MPa treatment and were not heat sensitized. Additionally, *B. subtilis* spores lacking all inner membrane GRs germinated extremely poorly in response to 150 MPa (Paidhungat et al. 2002). Under these moderate pressure and temperature conditions, significant spore inactivation (up to 4 $\log_{10}$ for *B. subtilis* and other spore strains) was achieved for pressure dwell times up to 24 h (Reineke et al. 2012). However, because individual spores (so-called superdormant spores that lack GRs) do not respond to this treatment, this process is not suitable for food sterilization (Zhang & Mathys 2019). Superdormant spores rarely or very slowly germinate in response to germination triggers and are the main limitation preventing industry from applying milder germination-inactivation strategies (Ghosh & Setlow 2009, Gould et al. 1968, Lovdal et al. 2011). This opens a research field of significant importance for the food industry. The relevance of superdormant spores is not only for HP-driven spore inactivation. A deeper understanding of the reasons for nutrient and HP superdormancy and for spores with a very slow germination rate is crucial for food safety. This information could enable a multihurdle sterilization concept for minimally processed food that reduces spore resistance properties by triggering spore germination (Zhang & Mathys 2019) or physicochemical hurdles, such as nisin or lysozyme; thus, the molecular targets of these spores are similar to those of vegetative cells, allowing a lower process intensity to be applied.

Dynamic ultra-high-pressure homogenization. The second possibility for applying HP to pumpable foods with limited particle size and concentration is ultra-high-pressure homogenization (UHPH). Herein, dynamic pressure and heat plus cavitation, shear stress, and impingement are combined to inactivate bacterial endospores (Sevenich & Mathys 2018). Pressure levels used are in excess of 300 MPa (Dumay et al. 2013) and hence much higher than in conventional homogenizers (≤50 MPa). With recent progress in pump and valve design, pressures of 450 MPa are

possible (New ETH Zurich prototype; Alexander Mathys, personal communication). The exact mechanisms leading to spore inactivation under dynamic HP treatment are not entirely understood (Dumay et al. 2013, Sevenich & Mathys 2018), but it is presumably due to a combination of the physical effects of pressure and temperature. During this process, valve temperatures of 120–155°C can occur but for less than 1 s, with possible similar impacts on spore inactivation as wet heat during USS (**Figure 1**), as described above (Mathys 2018). Very high temperatures after homogenization can be achieved only if the treated product is preheated. The pressure level and the homogenizer valve geometry can increase the product temperature by 18–23°C per 100 MPa of applied pressure (with final pressures up to 400 MPa) (Sevenich & Mathys 2018). Most of this temperature increase is caused by the conversion of kinetic energy into heat, plus additional ~3°C per 100 MPa (for water) adiabatic heat of compression. In the literature, spore-inactivation levels between 2 and 6 $\log_{10}$ are reported for several *Clostridia*, *Geobacillus*, and *Bacillus* strains (Cavender 2011, Dong et al. 2015, Georget et al. 2014b), but the inactivation mechanisms are not fully understood, with most authors attributing the inactivation to purely thermal inactivation (Sevenich & Mathys 2018). For high treatment temperatures (preheating to >80°C and valve temperatures of >140°C) and pressures above 300 MPa, thermal inactivation seems to be the major driving force for spore inactivation. The supposed impact of these HPs and temperatures on the inner spore membrane and subsequent release of Ca^{2+}-DPA (Georget et al. 2014b) remains to be shown and is likely superimposed by the effects of wet heat on spore core proteins.

Electromagnetic Inactivation Technologies

Another category of inactivation technologies used for food preservation is the electromagnetic inactivation technologies. This category includes a broad range of different emerging technologies with the potential to inactivate bacterial spores, including thermal treatments in which volumetric heating of food is achieved by resistance heating or in a microwave field, technologies combining heat and pulsed electric fields (PEF), and technologies utilizing different kinds of nonthermal irradiation or, notably, cold atmospheric plasma, which is a combination of UV irradiation and chemical inactivation with highly reactive species.

Pulsed electric fields. Contrary to resistance heating, also known as ohmic or Joule heating, where a constant electric field is used for a rapid volumetric heating of food, the application of pulsed electric fields (PEFs) aims to keep the product temperature as low as possible by applying electric current in an alternating electric field in a μs range. Current applications of PEF in the food industry aim to reduce the cutting force and/or to enhance mass transfer/press yield for fruit and vegetable products. Furthermore, some industrial-scale units are used for pasteurization of liquid foods at moderate temperatures (Mathys et al. 2013).

However, because an electric current is flowing through the treated product at several kHz, an energy input–dependent increase in temperature of the treated liquid food occurs, which is known as the Joule effect (Spilimbergo et al. 2003). Furthermore, the heterogeneity of the electric and flow fields in the PEF treatment chamber impacts the energy and temperature distribution in the flowing product (Buchmann et al. 2018a,b; Knoerzer et al. 2012; Meneses et al. 2011). On the basis of numerical simulations, temperature differences of 30–50 K between the PEF treatment electrodes and the flow liquid in the center of the treatment chamber are possible (Meneses et al. 2011). These temperatures are obtained only for very short treatment times (Meneses et al. 2011) but can exert a significant impact on endospore inactivation, as described for USS in the section titled Thermal Inactivation and Emerging Ultra-Short Sterilization. Inactivation of vegetative microorganisms by PEFs is well understood and is explained in detail by Jaeger et al. (2014).

Regarding spore inactivation mechanisms, only limited data are available and only for *Bacillus* and *Geobacillus* species so far, and the data are quite contradictory. Some authors reported no effect of PEF on spore inactivation (Devlieghere et al. 2004, Knorr et al. 2011, Wouters et al. 2001), whereas others stated at least a partial contribution of PEF on spore inactivation (Reineke et al. 2015b, Siemer et al. 2014) or very efficient inactivation of certain strains, such as *B. cereus*, at nearly ambient temperature (40°C) with a field intensity of 25 kV/cm and 20 pulses (Spilimbergo et al. 2003). Similar findings were also reported by Marquez et al. (1997) for *B. subtilis*, but all these studies were performed under different treatment conditions, hampering a direct comparison of the data to draw conclusions for spore-inactivation mechanisms. Reineke et al. (2015b) coupled spore-inactivation kinetics with the temperature field in a PEF cell and could not find a synergistic effect for *B. subtilis*, leading to the assumption that it is a pure thermal effect with underlying inactivation mechanisms as described above for USS treatments (**Figure 1**). In contrast to *Geobacillus stearothermophilus*, for higher preheating (95°C) with accordingly higher treatment temperatures (126°C), a beneficial effect of PEF was shown, producing a 3.2-$\log_{10}$ inactivation compared to 0.2 $\log_{10}$ for the thermal reference.

Somavat et al. (2012) related their accelerated inactivation of *G. stearothermophilus* spores in an ohmic heating system to the electric field frequency, and this was also found by Reineke et al. (2015b) for PEF. Furthermore, they hypothesized vibration of Ca^{2+}-DPA in the spore core (Somavat et al. 2012), implying higher resistance heating of the spore compared to the surrounding fluid. However, the heat flux of the high surface:volume ratio of an individual spore in a colder liquid challenges this hypothesis.

In addition, permeabilization of the inner spore membrane, which is crucial for spore survival, is questionable because its diameter is 4–12-fold smaller compared to a vegetative cell, and, hence, the critical electric field strength for irreversible pore formation is high. Nevertheless, PEF treatment causes significant alterations in spore morphology. Pillet et al. (2016) treated *Bacillus pumilus* spores with PEF (1,000 pulses with maximum 7.5 kJ) and reported partial destruction of the coat proteins but no impact on spore stiffness or volume, leading to the assumption that neither Ca^{2+}-DPA release nor permeabilization of the inner spore membrane occurred.

This impact on the outer spore membrane enables a promising multihurdle approach using lysozyme (E1105), an enzyme that catalyzes the hydrolysis of muramic acid and can decompose the spore cortex and germ cell wall. Lysozyme cannot penetrate an intact spore coat (Dirks 1999) but presumably could do so after an intense PEF treatment, which requires further investigation. Furthermore, essential oils (Gayán et al. 2012), sucrose laureate (Stewart et al. 2000), and polyphenols enhance spore inactivation in combination with HPP or PEF (Soni et al. 2016). For some essential oils, it is reported that they bind to the GRs of spores, and the bacteriocin nisin can induce pore formation in cell membranes (Aouadhi et al. 2014).

Ultraviolet radiation. The radiation dose of UV photons is expressed in J/m^2 or radiant energy received per unit area, and the germicidal wavelengths of the UV light used in the food industry are in the UVC spectrum from 200–280 nm (Soni et al. 2016). UVC photons have a photon energy of 4.43–6.20 eV, which is 100- to 10^7-fold less energy compared to electron beam treatment.

Compared to vegetative cells, bacterial spores are 10- to 50-fold more resistant to UV radiation (Setlow 2006). Knudson (1986) reported a D-value of 60 J/m^2 for vegetative *B. anthracis* compared to 810 J/m^2 for its spores, with the highest sporicidal effect at 254 nm (Nicholson et al. 2000). With longer UV wavelength, the sporicidal effect decreases (Setlow 2006), as well as the photon energy, whereas vacuum-UV (VUV) photons have a shorter wavelength (100–200 nm) and a higher photon energy (6.20–12.4 eV).

UV-photon-absorbing pigments can contribute to spore resistance against UV radiation and are located in the outer spore layers (Nicholson et al. 2005), but these pigments are not present in *Bacillus* spores (Setlow 2006). Hence, this protective mechanism is present only in a few spore strains. Spore DNA is the major lethal target of UVC (**Figure 1**), which causes single- and double-strand breaks, as well as base modification (Setlow 2006, Soni et al. 2016). If the DNA of vegetative cells is radiated with 254-nm lethal cyclobutane dimers, 6–4 photoproducts (64 pp) are formed. By far the dominant photoproduct in a spore is a thymidyl-thymidine adduct, also called the spore photoproduct (SP), which is less lethal than 64 pp and is repaired in the first minutes of spore outgrowth by at least three repair mechanisms (Setlow 2006), owing to the activity of photolyases (DNA repair enzymes) (Setlow & Li 2015). Although repair of SP occurs during spore outgrowth, the DNA in the dormant spore is protected by SASPs, low water content, and Ca^{2+}-DPA, which is a strong photosensitizer (Douki et al. 2005, Setlow 2006). The saturation of spore DNA with α- and β-SASPs is a major factor suppressing the formation of 64 pp that promotes the formation of SP (Setlow 2006).

Hence, the most efficient inactivation by UVC could be achieved by increasing the number of photons with a wavelength of 254 nm. One promising technical solution could be an Excimer UV laser with 248-nm wavelength and a high-photon fluence, which achieves a 5-$\log_{10}$ reduction in *B. cereus* spores on packing material without significant temperature increase (Soni et al. 2016).

Cold atmospheric pressure plasma. A promising surface decontamination technology for packing material, as well as food, is cold atmospheric pressure plasma (CAPP). Plasma is the fourth state of matter and is an at least partly ionized gas, which is described in detail by Hertwig et al. (2018). The composition of the plasma gas is highly dependent upon the kind of plasma system (plasma jet, dielectric barrier discharge, or corona discharge), the used plasma feed gas, the moisture content of the gas, and whether gas from the surrounding atmosphere in which the plasma was ignited interacts with the plasma itself (Bourke et al. 2017, Niemira 2012). The composition of CAPP can be complex and can consist of reactive oxygen species (ROS), such as atomic oxygen, ozone, singlet oxygen, and superoxide anions; reactive nitrogen species (RNS), such as nitrogen oxides; and reactive hydrogen species (RHS), such as hydrogen radicals, hydroxyl radicals, and hydrogen peroxide (Hertwig et al. 2018), as well as UV or VUV photons if the plasma and the treated surface are under vacuum (Brandenburg et al. 2009).

Regarding microbial and spore inactivation, the general underlying inactivation mechanisms of CAPP are difficult to describe, as the plasmas used in different studies are often not comparable. The systems differ in their mode of plasma generation, technical setup, energy converted into plasma, gas or gas composition, moisture content in plasma close to the treated surface, application time, and temperature. Hence, further studies are needed with comparable and standardized treatments. As most systems have at least a few similarities, it can be concluded that if UV photons are present in the plasma, they can cause DNA damage as described above (**Figure 1**). Increased spore inactivation in response to increased UV emission was demonstrated by Boudam et al. (2006) and Reineke et al. (2015a). Hertwig et al. (2015) proved that this correlates with increased DNA damage.

Hertwig et al. (2017) treated different *B. subtilis* mutant strains and showed that the outer spore coat, Ca^{2+}-DPA, and SASPs contributed to CAPP resistance. Given that the plasma used in this study also emitted UV photons, these findings are in line with spore resistance to UV radiation, as described above.

Additionally, UV photons can break covalent chemical bonds, resulting in atom erosion of the organic material, which is called intrinsic photodesorption (Moisan et al. 2001). Moisan et al. (2001) further described a second inactivation mechanism of relevance for spore inactivation,

called etching (**Figure 1**). Continuous bombardment with atoms, molecular radicals, and excited molecules similar to photodesorption causes erosion of a spore layer by layer, which was shown for singlet oxygen atoms and radicals by Park et al. (2003). Wang et al. (2016) treated *B. subtilis* spores with CAPP with and without UV shielding and analyzed individual spores with phase-contrast microscopy and Raman spectroscopy. For short treatments, they reported 2-$\log_{10}$ inactivation and delayed germination. Prolonged treatment up to 10 min caused a 5-$\log_{10}$ inactivation and Ca^{2+}-DPA release. The investigators concluded charged particles and ROS cause damage to key germination proteins (short CAPP exposure) and the inner spore membrane.

Given that the CLEs in *B. subtilis* are located between the outer coat and the cortex (CwlJ) as well as between the cortex and inner spore membrane (SleB), and the GRs are located on the inner spore membrane, prolonged CAPP treatments erode the inner spore membrane by etching, causing Ca^{2+}-DPA release and spore inactivation. These three mechanisms explain the often-reported biphasic inactivation of bacterial spores inoculated on a smooth surface and treated with CAPP.

Of interest in this regard is the formation of nitrous acids, which are formed on wet food surfaces in contact with NO_x from CAPP or in plasma-activated water. Strong acid treatment can inactivate spores by rupturing of the inner spore membrane (Setlow 2006), also known as "pop" opening because of the sudden expansion of the compressed spore core. Furthermore, hydrogen peroxide reacts with lipids in the cell wall and DNA (Setlow 2006). In an aqueous media, spores can also take up hydrogen peroxide, which is cleaved into hydroxyl ions in the presence of UV photons, which further react with spore DNA, causing spore death (Soni et al. 2016).

Electron beam. In general, food irradiation is the treatment of foodstuff with a certain type of radiant energy known as ionizing radiation. In 1981, the World Health Organization (WHO 1981) established 10 kGy as the maximum dose for food processing without any adverse effect on food matrices and more recently concluded that no limiting dose is required (WHO 1999). Radiant energy applied for food treatment exhibits different wavelengths and degrees of power, disappearing when the energy source is removed and classified in four different technologies based on wavelength and energy intensity. Irradiation includes γ-rays, X-rays, electron beams, and UV photons. The achievable energy density for X-rays in large-scale facilities with the potential to irradiate food is limited and, hence, they are not used for preservation. Electron beams, γ-rays, and UV photons belong to the high-energy ionizing irradiation group, with UV-photons having the lowest energy (Hertwig et al. 2018, Soni et al. 2016). The high kinetic energy of γ-rays enables treatment of a broad range of food, whereas the penetration depth of electrons emitted by an electron beam depends on its kinetic energy. In this regard, electron beams are classified into high-energy (>300 keV) and low-energy (<300 keV) (Hertwig et al. 2018) beams. This distinction enables a controllable penetration of the electrons into the food from a few micrometers, for low energy electrons, to a few centimeters, for high energy electrons (Zhang et al. 2018). Hence, a low-energy electron beam (LEEB) emits electrons close to the surface of treated food and could be a very promising technology for decontamination of food surfaces, seeds, and powders (Hayashi et al. 1997, 1998; Zhang et al. 2018). As for all described inactivation technologies, bacterial spores exhibit high resistance to irradiation compared to vegetative microorganisms (Chalise et al. 2007, Rahman et al. 2006, Setlow 2006, Tahergorabi et al. 2012). Low core-water content is presumably the most important factor (Setlow 2006) for increased resistance, as the direct alteration of a high-energy electron beam (HEEB) with atoms results in the formation of radicals. The expulsion of electrons is primarily affected by the presence of oxygen and water, underlying the major effect of the physiological state of the spore and its corresponding core-water content (Fiester et al. 2012). Consequently, spore resistance to HEEB is increased in very low water environments, such as in packaging material. The photons and electrons are quenched, and their ability to generate

damaging hydroxyl radicals is reduced (Setlow 2006). Furthermore, Nicholson et al. (2000) showed that α- and β-SASPs are at least involved in spore resistance to γ-irradiation. However, along with the impact of Ca^{2+}-DPA, which contributes to spore UV resistance, this must be confirmed. On the basis of the ability of ionizing radiation to form hydroxyl radicals (OH-), as well as hydrogen peroxide (H_2O_2) and other reactive species in high water environments, the water content and state of the spore, dormant or germinating, are important, as these reactive species can react with nucleic acids (Setlow 2006). Hence, the free water in not only the spore core but also the surrounding medium affects spore inactivation, explaining the often reported increased resistance of spores in frozen media (Black & Jaczynski 2006, Sommers & Fan 2013).

Consequently, the primary target for spore inactivation by ionizing radiation is DNA (Moeller et al. 2008, Nicholson et al. 2000, Setlow 2006), which is vulnerable to single- and double-strand breaks and base modifications (**Figure 1**); it is assumed that this is also the mode of inactivation for high-energy electrons (Chalise et al. 2007). High-energy photons and electrons both directly and indirectly interact with spore DNA, RNA, enzymes, and membrane proteins, and lethality occurs through accumulation of all these interactions. As DNA is the most sensitive target for ionizing radiation, differences in resistance between spore species are typically very small (Gayán et al. 2014). The formation of ROS, RNS, and RHS during HEEB irradiation can further cause loss of spore coat integrity (Fiester et al. 2012), which likely has a similar effect as etching and erosion rather than the lethal effect of reactive species formed inside the spore. Furthermore, Fiester et al. (2012) measured the release of cytoplasmic material at HEEB doses insufficient to substantially cause DNA strand breaks and attributed the damage to spore coat and membrane damage in general. The reported uptake of the nonmembrane permeable fluorescent dye propidium iodide (PI) (Fiester et al. 2012), which stains the DNA, strongly indicates that the inner spore membrane is also affected by reactive species, leading to subsequent spore inactivation.

For LEEB, lower resistance was reported for gram-negative *E. coli* than for vegetative gram-positive *B. subtilis* (Chalise et al. 2004, Rahman et al. 2006). The reasons for this could be multifold, but an important difference between treatments with high- and low-energy radiation is the formation of a so-called electron cloud. On the way to the treated surface, primary low-energy electrons generate secondary electrons with lower energy through inelastic collision (Hertwig et al. 2018). These secondary electrons can damage DNA as well (Brun et al. 2009, Nikjoo & Lindborg 2010, Zhang et al. 2018) but with limited penetration depth. Ghomi et al. (2005) tested the impact of LEEB (80 keV) on the inactivation of different *E. coli* concentrations and reported decreasing inactivation with increasing cell concentrations, which they attributed to the formation of agglomerates. The agglomeration of spores could be an issue (Mathys et al. 2007b), which could also impact spore inactivation because of very low kinetic energies with small penetration depths. Furthermore, the distance between low-energy electron emission to the atmosphere and the spore or spore agglomerates does impact its lethality because of collisions with atmospheric molecules (Hertwig et al. 2018) and surface morphology (Fan et al. 2017). Zhang et al. (2018) showed in their study on LEEB inactivation that *G. stearothermophilus* ATCC 7953 spores were the most LEEB-resistant strain (with D-values of 1.65 kGy at 80 keV and 2.35 kGy at 200 keV) among several *Bacillus* species, including *B. pumilus* DSM 492, a biological indicator for irradiation-based sterilization (Prince 1976). After HEEB treatment with 10 MeV of *B. pumilus* DSM 492, varying D-values, e.g., 1.54 kGy (Tallentire et al. 2010), 2.12 kGy (Urgiles et al. 2007), and 2.64 kGy (Pillai et al. 2006), were published by several groups. The dimensionless value of the relative biological effectiveness (RBE), which converts the absorbed dose for radiation into a biological equivalent dose, may be suitable to compare these different kinds of radiation with regard to their antimicrobial effects (Hertwig et al. 2018). Higher RBEs are more damaging, and Bellamy et al. (2015) reported an RBE > 1 for LEEB treatment compared to a reference electron radiation of 1 MeV.

CONCLUSIONS

Like endospore inactivation by heat only, it is generally difficult to identify one particular target structure, molecule, or enzymatic system that is responsible for spore inactivation by each of the above-discussed technologies. The structure, defense, and germination mechanisms between different spore strains, or even between *Bacillus* and *Clostridium* spores, have many similarities, and there are different levels of protection and resistance for each of the described technologies. Consequently, different sterilization indicator strains, which have yet to be identified for emerging preservation technologies, are needed for each of the technologies. Further research, especially for *Clostridium* spores, is needed to confirm or challenge the defense and inactivation pathways described above, which are often based only on data for *B. subtilis*. Taking this information into account and combining it with the knowledge about different molecular or cellular structures in endospores, including superdormant spore fractions, a tailored inactivation concept is possible. In this perspective, a science-based combination of different physical preservation technologies and physicochemical hurdles may lead to multihurdle sterilization for minimally processed food.

DISCLOSURE STATEMENT

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