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REVIEW ARTICLE

High hydrostatic pressure-induced inactivation of bacterial spores

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Abstract

High hydrostatic pressure (HHP) is the most-widely adopted novel non-thermal technology for the commercial pasteurization of foods. However, HHP-induced inactivation of bacterial spores remains a challenge due to spore resistance to the treatment limits of currently available industrial HHP units (i.e. ~650 MPa and 50 °C). Several reports have demonstrated that high pressure can modulate the germination machinery of bacterial spores, rendering them susceptible to subsequent inactivation treatments. Unfortunately, high pressure-induced germination is a unique phenomenon for spores of the genus *Bacillus* but not of *Clostridium*. Alternative strategies to inactivate bacterial spores at commercially available pressure and temperature levels include promoting the germination step by inclusion of known germinants into the food formulation to increase the lethality of HHP treatments on bacterial spores. The aim of this review is to provide an overview of the molecular basis involved in pressure-triggered germination of bacterial spores and of novel strategies to inactivate bacterial spores with HHP treatments.

Keywords

Bacterial spores, HHP, PATPS, spore inactivation, spore germination

History

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Introduction

New consumer demands for chemical additives reductions and minimal processing of foods have raised a need for the development of novel processing technologies to produce foods that approach absolute chemical and microbial safety when consumed. High hydrostatic pressure (HHP) processing is a novel technology alternative that meets these consumer demands to some extent while retaining the sensory and nutritional quality of freshly prepared foods. HHP processing has been the most successful alternative novel technology adopted by the food industry, and it is used primarily for the pasteurization of refrigerated low-acid foods.

Industrial HHP processing relies solely on elevated pressure (~400–600 MPa) treatments at refrigerated or room temperature (~4–25 °C) to increase shelf-life, while retaining fresh-taste and reducing microbial loads to levels similar to those achieved by thermal pasteurization (Mújica-Paz et al., 2011; Pérez Lamela & Torres, 2008; Torres et al., 2009; Torres & Velazquez, 2008). These processing conditions inactivate non-spore forming food-borne pathogens; however, HHP processed foods, albeit free of vegetative cells, retain nearly unaltered pathogenic and food spoilage bacterial spore levels found in raw foods (Gayán et al., 2012). The recent development of pressure-assisted thermal processing (PATP) units, operating above 600 MPa and 100 °C, enables high-pressure technology to meet demands for commercial sterilization of food products, while exerting a low impact on their functional properties and nutritional value (Pérez Lamela & Torres, 2008). Although commercial PATP prototypes are currently available from Avure Technologies Inc. (www.avure.com, Franklin, TN) and NC Hyperbaric Inc. (www.hyperbaric.com, Burgos, Spain), industrial scale HHP units reach only ~50 °C, i.e. temperature levels with no bacterial spore inactivation effect. This highlights the need for further research on combined strategies to inactivate bacterial spores in food products at currently feasible pressure and temperature levels.

Knowledge on the spore germination process has increased considerably during the last two decades, particularly with respect to the molecular understanding of the mechanisms of germination of *Bacillus* spores (Moir, 2006; Paredes-Sabja et al., 2011; Setlow, 2003). This has allowed the development of novel strategies to inactivate bacterial spores in a two-step process, i.e. germination followed by inactivation (Akhtar et al., 2009; Black et al., 2008). Bacterial spores lose their inactivation resistance properties upon germination, and they can be inactivated by milder pressure and temperature conditions (Akhtar et al., 2009; Black et al., 2008). In addition, pressure-induced germination strategies can be combined with natural compounds with known antibacterial properties such as bacteriocins and essential oils added during food formulation (Gayán et al., 2012). These hurdles to the survival of bacterial spores represent viable strategies for the production of low-acid ($pH > 4.5$) shelf-stable foods

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relying on HHP treatments. These developments in the HHP technology, combined with advances in understanding the mechanisms of bacterial spore resistance and germination, justify the need to summarize the current knowledge of these molecular mechanisms to help identify novel strategies to inactivate bacterial spores by milder HHP treatments, which is the aim of this review.

Resistance of relevant endospore-forming bacterial species to HHP

The development of HHP processing needs to ensure that the technology achieves efficient inactivation of pathogenic and spoilage food-borne microorganisms (Lado & Yousef, 2002). Due to their intrinsic pressure-resistance, inactivation of bacterial spores remains a priority when developing strategies to extend the product's shelf-life, or to achieve a certain food safety level. Two types of bacterial spores may be found in foods and food processing environments, those from pathogenic or food spoilage bacterial species.

Pathogenic food-borne spore-forming bacterial species

Three major pathogenic bacterial spores of food safety concern are used as target organism when developing food processing treatments

(i) *Clostridium botulinum*, a microorganism indigenous to soil and water and considered a bioterrorism agent due to its ability to produce a potent neurotoxin causing muscular paralysis, is the primary pathogen that food sterilization protocols must eliminate (Jay et al., 2005). HHP treatments at 827 MPa and 75 °C barely yield ~3 decimal reductions (DR) in *C. botulinum* spore counts (Reddy et al., 2003). In addition, *C. botulinum* spore's resistance to high pressure in low-acid foods is type-dependent, with type A spores being more sensitive than type B spores (Margosch et al., 2004).

(ii) *Clostridium perfringens* causing more than one million cases of *Clostridium perfringens* type A food poisoning cases in the USA alone, ranks as the second most commonly reported bacterial food-borne disease (Scallan et al., 2011). HHP treatments of *C. perfringens* spores at 650 MPa and 75 °C yields ~1 DR (Paredes-Sabja et al., 2007). There is also a lack of correlation between heat- and pressure-resistance within *C. perfringens* spores, as *C. perfringens* isolates with the most heat-resistant spores do not produce the most pressure-resistant spores (Paredes-Sabja et al., 2007).

(iii) *Bacillus cereus* is recognized as the main cause of food poisoning in several countries, and is typically associated with low-acid foods with limited refrigerated shelf-life. Similar to the aforementioned spore formers, no significant inactivation of *B. cereus* spores is observed at pressure levels of ~500–600 MPa (Marco et al., 2011).

Food spoilage spore-forming bacterial species

Spore-former species causing food spoilage have major financial consequences in the food industry, therefore efforts are made to develop strategies that also inactivate them.

Common species from the *Bacillus* genus found in low acid manufactured foods are *Bacillus subtilis*, *Geobacillus stearothermophilus*, *Bacillus licheniformis*, *Bacillus coagulans*, and *Bacillus pumilus* (Oomes et al., 2007). As in the case of *C. perfringens* spores, there is no correlation between their heat and pressure resistance, and in spite of the large variation in heat resistance, all Bacillus genus spores survive pressures of up to 940 MPa for 40 min at room temperature (Margosch et al., 2004). Another important spore species in the spoilage of high-acid foods, such as fruit and vegetable juices, is *Alicyclobacillus acidoterrestis* (Steyn et al., 2011). Although spores of *A. acidoterrestis* are slightly heat sensitive ($D_{90\,^{\circ}C}$ 15–23 min and $D_{95\,^{\circ}C}$ 2.4–2.8 min) (Steyn et al., 2011), these spores are not affected by treatment of 621 MPa and only 2 and 4 DR were observed when combining 45 or 95 °C, respectively, with treatments at 621 MPa for 10 min (Lee et al., 2006; Silva et al., 2012).

Food spoilage members of *Clostridiales* are of particular concern in vacuum-packed meat products and dairy products. Typical species implicated in food spoilage include: (i) *Clostridium sporogenes*, a non-pathogenic, spore-forming, anaerobic bacterium considered for safety reasons to be a non-toxigenic equivalent of proteolytic *C. botulinum* (types B, E, F) (Lund & Peck, 2000) and therefore regarded as a safe and suitable surrogate for *C. botulinum* (Montville et al., 1985); and (ii) *Clostridium frigidicarnis, Clostridium algidicarnis, Clostridium algidixylanolyticum, Clostridium esterheticum, Clostridium laramiense and Clostridium gasigenes*. These species are associated with spoilage of chilled red meat (Adam et al., 2010), and can grow between −5 and 20 °C (Adam et al., 2010). These species form heat-resistant spores capable of surviving a pasteurization process (Adam et al., 2011), leading to spoilage of meat products typically recognized as ''blown pack'' or surface spoilage and resulting in great economic losses (Adam et al., 2010). However, there is no available information on their pressure resistance.

Spore formation, structure, and resistance

Spore-forming bacterial species initiate the sporulation cycle upon sensing a variety of environmental signals that include cell density, nutrient starvation, and quorum sensing among others. These signals are detected by orphan histidine kinases that ultimately phosphorylate the master regulator of sporulation, the transcriptional regulator Spo0A (Losick & Stragier, 1992). This initiates a complex regulatory circuit orchestrated by four main RNA polymerase sigma factors (Losick & Stragier, 1992). SigF and SigG are specific to the forespore, while SigE and SigK are specific to the mother cell compartment (Losick & Stragier, 1992). The developmental process of sporulation in all spore-forming species studied typically lasts ~8 h culminating with the formation of a mature, dormant and fully resistant spore and a lysed mother cell (Paredes-Sabja & Sarker, 2009). This dormant spore can persist in the environment for an extended time.

Bacterial spores possess various structural component that aid in their resistance to environmental stresses, including the typical thermal processing treatments used in the food industry (Setlow, 2007). The outer most layer, the exosporium in some species, and the coats in the majority of spore-

forming species (Henriques & Moran, 2007) play little to no role in the resistance to heat treatments (Setlow, 2006), however its role in high-pressure resistance remains unclear. Underneath the coats is the outer membrane, which is presumably lost during long term dormancy and plays no role in spore resistance. Below the outer membrane is the spore peptidoglycan cortex that possesses a lower degree of crosslinking between the glycan strands than in the peptidoglycan cell wall of vegetative cells (Popham et al., 1999), providing the spore cortex with elastic properties that are directly linked to spore wet-heat resistance (Popham et al., 1995). Below the cortex is a layer of peptidoglycan (PG) cell wall with a structure presumably similar to that of growing cells that becomes the cell wall during spore outgrowth (Setlow, 2003). Beneath the germ cell wall is a compressed spore inner membrane of immobilized lipids (Moir et al., 2002) with low permeability to small molecules including water (Moir et al., 2002), providing protection from DNA damaging chemicals (Setlow, 2006). The innermost layer, the core with a low water content (20–50% of wet weight), contains RNA, the spore's DNA, most of the spore enzymes, and a large deposit of dipicolinic acid (DPA) chelated at a 1:1 ratio with divalent cations predominantly Ca^{+2} (Ca-DPA). These large amounts of Ca-DPA contribute to maintain a low core hydration, thus enhancing the spore's resistance to wet heat, UV radiation and chemicals (Setlow, 2006). The low hydration levels of the spore's core contribute to the binding of α/β-type small, acid soluble proteins (SASP) to the spore's DNA, saturating the DNA with SASPs and contributing to spore resistance (Paredes-Sabja et al., 2008b; Setlow, 2007). It is worth to note that an important factor contributing to the bacterial spore resistance and their ability to germinate are the conditions under which they sporulate (Minh et al., 2011; Paredes-Sabja et al., 2008a; Raso et al., 1998a). For example, sporulation at 42 °C yields *C. perfringens* spores that have higher heat resistance than those prepared at 37 °C (Paredes-Sabja et al., 2008a). Conversely, spores prepared at 27 °C had lower resistance than those prepared at 37 °C (Paredes-Sabja et al., 2008a). Although variables of sporulation conditions are carefully controlled in laboratory experiments, this is not possible for bacterial spores found in foods, and should be taken into account when analyzing the inactivation of spores in a food processing setting.

Spore germination and loss of resistance factors

The aforementioned spore resistance factors are quickly lost during spore germination, which can be triggered by the presence of germinants sensed through cognate germinant receptors (GRs) localized in the spore inner membrane (Paredes-Sabja et al., 2011; Setlow, 2003). The specificity of the GRs to different nutrients is dependent on the GR and the spore species (Paredes-Sabja et al., 2011). However, common nutrients triggering the germination of spores of several *Bacillus* and *Clostridium* species have been identified and include L-alanine, the mixture of L-asparagine, glucose, fructose and KCl, some nucleosides and salts (Paredes-Sabja et al., 2011). Binding of nutrients to their cognate receptors leads to an irreversible germination process characterized by the release of small molecules through a series of biophysical events. These include the release of the majority (>90%) of the spore core DPA content as a 1:1 chelate with divalent cations, predominantly Ca^{2+} (Kong et al., 2010; Wang et al., 2011). Ca-DPA is released within <3 min through specific DPA-channels localized in the spore's inner membrane (Vepachedu & Setlow, 2005, 2007). Other small molecules released during early germination include monovalent cations (i.e. H^+, Na^+ and K^+) through an energy independent process (Setlow, 2003; Swerdlow et al., 1981). These early events produce a partial increase in core hydration, which at least in *C. perfringens* leads to the dissociation of SASPs from the spore core DNA leading to a decrease in spore resistance (Paredes-Sabja et al., 2008b). In spores of *Bacillus* species, the release of Ca-DPA activates one of the cortex lytic enzymes (CLEs), CwlJ (Paidhungat et al., 2001), initiating the hydrolysis of the cortex. In parallel, the release of material from the spore core possibly produces a decrease in the constraint of the spore PG cortex activating another redundant CLE, SleB (Magge et al., 2008; Tovar-Rojo et al., 2002). Removal of the spore PG allows full core rehydration, resumption of enzymatic activity and spore outgrowth (Paredes-Sabja et al., 2011; Setlow, 2003). At this stage, the spore has lost all of its resistance properties and has become sensitive to mild heat and chemical stress. This chemically-induced germination process suggests the opportunity to fully release/degrade the bacterial spore resistance by activating its germination machinery during food processing, and thus rendering spores that can be inactivated by mild processing conditions.

HHP-inactivation strategies for bacterial spores

Low hydrostatic pressure induces germination of Bacillus spores

Spores of *Bacillus* species can initiate and go through the process of germination under relatively low pressure (i.e. 100–150 MPa) (Black et al., 2005). Treatments of 150 MPa for 7 min at 37 °C (Black et al., 2005) or 100 MPa for 30 min at 40 °C (Wuytack & Michiels, 2001) are sufficient to trigger germination of ~90% of the spore population without further incubation. In spores of *Bacillus* species, low pressure triggers germination through the GRs (Black et al., 2005). While GerA is the most responsive GR for low pressure-induced germination of spores of *B. subtilis* mainly due to its higher abundance (Black et al., 2005), absence of any single GR does not affect low pressure-triggered spore germination in *B. cereus* (Wei et al., 2009). Once a GR becomes pressure-activated, Ca-DPA is released from the spore core activating both major CLEs present in *B. sutbilis* spores (Black et al., 2007). The GerD lipoprotein localized in the spore's inner membrane (Pelczar & Setlow, 2008) forming discrete clusters with the GR (Griffiths et al., 2011) is perhaps involved in amplifying the nutrient-induced germination signal that is required for low pressure-induced germination of *B. subtilis* spores (Pelczar et al., 2007). In other species such as *B. cereus*, antiporters (i.e. GerN and GerT) involved in nutrient germination have been shown to be required for low pressure-induced germination (Wei et al., 2009). Although the mechanism of low pressure-induced germination of *B. subtilis* spores seems to be similar to that of nutrient-initiated

germination, including the resumption of metabolism (Wuytack et al., 1998), several differences indicate that low pressure modulate the GRs differently than the nutrient germinants. For example, diacylglycerol covalently added to a cysteine residue in the N-terminal domain of the GR C-subunit protein is essential for nutrient germination for all GRs in *B. subtilis* spores, yet it only seems to be essential for low pressure-induced germination through the GerA receptor but not for GerB and GerK receptors (Black et al., 2005).

Low pressure-induced germination conditions vary greatly between different species. In *B. subtilis*, optimum low pressure-induced germination conditions maximize at 40 °C and 65 °C in *B. cereus* (Wei et al., 2009). These differences are likely to be due to temperature sensitivity of a protein essential for germination (Wei et al., 2009), and should also be considered with other food-borne spore-former bacterial pathogens in order to optimize high pressure processing regimes.

Moderate pressure-induced germination of *Bacillus* spores

Beside low pressure-induced germination, moderately high pressures (i.e. ~500–600 MPa) also trigger the germination of spores of *Bacillus* species, but through a GR-independent pathway (Black et al., 2007). Evidence suggests that moderately high pressure triggers DPA release and other small molecules by acting on the DPA channels localized in the spore's inner membrane. *B. subtilis* spores with higher levels of SpoVA proteins released Ca-DPA at higher rates than wild-type spores after treatment with 500 MPa, indicating that the SpoVA proteins are involved in DPA-release induced by moderate pressures (Vepachedu et al., 2007). Indeed, while *B. subtilis* spores lacking all GRs were able to germinate upon moderate pressure treatment, *B. subtilis* spores lacking both CLEs (SleB and CwlJ) and DPA-less spores lacking SleB were not able to germinate (Black et al., 2007). This suggests that moderate pressure (i.e. 500 MPa) triggers DPA release and that this latter event then activates both CLEs in *B. subtilis* spores much like Ca-DPA activates the CLEs during nutrient germination under normal pressure conditions. This moderate pressure-induced germination mechanism has also been validated in spores of other *Bacillus* species (i.e. *B. cereus*), indicating that this strategy could be used to trigger germination of spores of at least some *Bacillus* species that possess a similar cortex hydrolytic machinery as that of *B. subtilis* and *B. cereus*.

Loss of spore resistance factors during pressure-induced spore germination of *Bacillus* species

Several studies suggest that pressure inactivates bacterial spores by a three-step model of inactivation, which involves a germination step followed by an inactivation step that compromises the spore's inner membrane (Mathys et al., 2007, 2009). The physiological changes in bacterial spores through high pressure are easily quantifiable by flow cytometry and staining with fluorescent SYTO 16 dye (Mathys et al., 2007). As mentioned earlier, low and moderate pressure-induced germination not only leads to different spore germination pathways, but also to significant differences in the loss of factors involved in thermal resistance between these two pressure-germination pathways (Coleman et al., 2007; Setlow, 2007). Both pressure levels trigger the release of Ca-DPA from the spore core and the degradation of the spore PG cortex, which are important factors in thermal resistance. However, a significant difference is that at low, but not at moderate pressure-induced germination, *B. subtilis* spores degrade their SASPs, likely via the activation of the germination protease involved in degradation of SASP and germination protease (GPR) (Wuytack et al., 1998). This might explain why low pressure-germinated spores of *B. subtilis* are more easily inactivated than those germinated at 500 MPa, to a subsequent pressure treatment of 600 MPa at 40 °C (Wuytack et al., 1998). This also suggests that SASPs, but not DPA or the integrity of the spore's PG cortex, might play a role in the resistance to pressure with no heating. Despite the lack of degradation of SASPs in *B. subtilis* spores germinated at 500 MPa, these spores were sensitive to UV and hydrogen peroxide treatments (Wuytack et al., 1998).

Modulation of the loss of spore-resistance factors can be achieved by changing the pressure-temperature conditions. However, caution should be taken, as most of the enzymes involved in germination might be inactivated after a certain pressure threshold, which might be species dependent. For example, it is tempting to speculate that the GPR protease that degrades SASPs only under low pressures (~100–150 MPa), but not under moderate pressures, might become inactivated at moderate pressures in *B. subtilis* spores. Similarly, under moderate pressures, CLEs of *B. subtilis* spores become activated after Ca-DPA release, degrading the spore PG cortex under moderate pressure conditions. However, in the case of *B. subtilis*, CLEs become inactivated at pressures higher than 700 MPa, or 600 MPa combined with temperatures higher than 70 °C (Reineke et al., 2011).

Synergistic effect of HHP and temperature on spore inactivation

Despite the great wealth of knowledge of spore resistance to heat treatments, studies on the factors involved in spore resistance to HHP are limited. Few studies (Black et al., 2005; Paidhungat et al., 2000; Paredes-Sabja et al., 2008b; Reineke et al., 2011) provide evidence that the mechanism by which spores survive HHP is significantly different to those involved in wet-heat resistance. Several lines of evidence come from studies in both *Bacillus* and *Clostridium* species. For example, DPA in the spore core is required for the resistance of *B. subtilis* and *C. perfringens* spores to wet heat (Paidhungat et al., 2000; Paredes-Sabja et al., 2008b). However, upon pressurization of *B. subtilis* spores with 550 MPa at 37 °C for 120 min, Ca-DPA is completely released from the spore's core, but no spore viability loss is observed (Black et al., 2005; Reineke et al., 2011), indicating that pressurization of the spore core that lacks DPA does not induce inactivation of essential proteins for spore viability. This phenomenon further suggests that since a partial core hydration follows DPA release under high-pressure conditions, the genetic material and essential enzymes inside a partially

Table 1. Effect of high hydrostatic pressure (HHP) treatments on the inactivation of food relevant pathogenic and spoilage bacterial spores.

Strain	Food matrix	Treatment	Germination step	DR(*)	References
Bacillus cereus	Milk	200 MPa, 45 °C, 30 min + 60 °C, 10 min	Yes	6.0	Van Opstal et al. (2004)
		600 Mpa, 60 °C, 30 min	No	6.0	
Clostridium botulinum	THB (pH 5.15)	600 MPa, 70 °C, 8 min	No	0.5	Margosch et al. (2006)
		600 MPa, 90 °C, 8 min	No	1.0	
		600 MPa, 110 °C, 8 min	No	4.0	
Clostridium perfringens	0.1 M citric acid (pH 6.5)	550 MPa, 55 °C, 15 min	No	0.4	Paredes-Sabja et al. (2007)
		650 MPa, 75 °C, 15 min	No	1.0	
	Ground poultry	568 MPa, 55 °C, 30 min	No	0.5	Akhtar et al. (2008)
		80 °C, 10 min + 568 MPa, 70 °C, 15 min	Yes	4.0	
Acidobacillus acidoterrestris	Apple juice concentrate (30 ° Brix)	621 MPa, 22 °C, 10 min	No	0	Lee et al. (2006)
		621 MPa, 45 °C, 10 min	No	2	
		621 MPa, 71 °C, 10 min	No	5	
		621 MPa, 90 °C, 10 min	No	>5	
	Tomato sauce (pH 4.2)	200 MPa, 20 °C, 10 min + 80 °C, 10 min	Yes	5	Vercammen et al. (2012)
		600 MPa, 25 °C, 10 min	No	0	
		600 MPa, 60 °C, 10 min	No	4	
Bacillus coagulans	Tomato sauce (pH 4.2)	200 MPa, 20 °C, 10 min + 80 °C, 10 min	Yes	0.3	Vercammen et al. (2012)
		800 MPa, 25 °C, 10 min	No	0	
		800 MPa, 60 °C, 10 min	No	1	
Clostridium sporogenes	0.1 M citric acid (pH 6.5)	550 MPa, 55 °C, 15 min	No	0.5	Paredes-Sabja et al. (2007)
		650 MPa, 75 °C, 15 min	No	2.1	

(*) DR, decimal reduction.

hydrated spore core lacking DPA is able to withstand the pressure stress. These observations would clearly indicate why at least *Bacillus* spores can germinate and remain viable at pressure levels of ~550 MPa (Black et al., 2005; Reineke et al., 2011).

The pressure-triggered DPA-release from the spore core would further explain the synergism between temperature and pressure. Several studies (Ramaswamy et al., 2010; Ramaswamy & Shao, 2010) have shown that when pressures higher than 700 MPa are combined with temperatures between 80 and 100 °C, *D*-values can be reduced by 6 to 10-fold. This increase in lethality indicates that there must be targets that are being affected by these pressure-temperature combinations. In fact, a recent study (Wang et al., 2012) has identified some potential targets of bacterial spores that are inactivated by heat such as the serine protease CspB that activates CLE SleC (i.e. the sole essential CLE) of *C. perfringens* spores, and the proteins that are involved in the release of DPA from the spore core (Wang et al., 2012). Several examples of the synergistic effects of the combination of high temperatures and pressure can be observed in Table 1.

High pressure-induced germination of spores of *Clostridium* species, does it really work?

In spite of the extensive understanding of the mechanism of spore germination in *Bacillus* species, much less is known on *Clostridium* spores. However, some very recent studies (Akhtar et al., 2009; Paredes-Sabja et al., 2008b; 2009a,b,c,d,e; Paredes-Sabja & Sarker, 2010) have contributed to significant progress. Most importantly, unlike spores of *Bacillus* species encoding 4–8 different ABC tricistronic GR operons, the sequenced genome of *Clostridium* species encodes a lower number of non-tricistronic GRs (Xiao et al., 2011). Indeed, functional studies in *C. perfringens* SM101 spores have shown that proteins encoded by a bicistronic *gerKA-KC* operon are essential for L-asparagine, Ca-DPA, K^+ and inorganic phosphate as well as for the viability of *C. perfringens* spores (Paredes-Sabja et al., 2008c; 2009c, e). In striking contrast to *B. sutbilis* spores, activation of the cortex hydrolysis machinery in *C. perfringens* spores follows a different mechanism that can have major implications in the development of pressure treatments to trigger germination. First, in *C. perfringens* spores, Ca-DPA acts through the GerKA-KC receptor pathway and does not directly activate the cortex lytic machinery (i.e. CspB and SleC [CS]) (Paredes-Sabja et al., 2008c; 2009b, d). Secondly, *C. perfringens* spores that lack DPA are stable and are able to germinate as wild-type spores, indicating that a reduction of the stress of the cortex constrain during DPA release is not required to activate the CS cortex hydrolytic machinery (Paredes-Sabja, Setlow et al., 2008b). The latter findings imply that it is unlikely that moderately high pressures (~500–600 MPa) will activate the CS cortex hydrolytic machinery through a DPA-dependent pathway as in *Bacillus* spores (Black et al., 2007). Thirdly, low pressures (~100 and 150 MPa) do not induce germination in *C. perfringens* SM101 spores within 60 min after pressure treatment (Akhtar et al., 2009). Similarly, a study using spores of pathogenic *C. perfrigens* 1027 and food spoilage *Clostridium laramie* treated with pressures from 138 to 483 MPa at 50 °C, reported ~50% of germination when subsequently incubated at 25 °C for 24 h, but this germination response is too slow for the development of efficient pressure-inactivation strategies (Kalchayanand et al., 2004). In agreement with this theory, the only species of *Clostridium* genus capable of germinating

after a pressure treatment is *C. sporogenes*, whose cortex lytic machinery resembles that of *B. subtilis* (Paredes-Sabja et al., 2011). *C. sporogenes* spores have been shown to initiate germination with low pressures (~100–200 MPa) or moderate pressures (~400–600 MPa) at either 40 or 60 °C (Mills et al., 1998).

When developing pressure-induced germination protocols, food processors seek a fast spore germination response after pressure treatments to rapidly achieve a high spore inactivation level (4–6 DR) by a second inactivation treatment, while avoiding microbial growth. In this context, pressure-induced spore germination studies (Akhtar et al., 2009; Kalchayanand et al., 2004) suggest that species with CS cortex hydrolytic machinery similar to that of *C. perfringens* are unlikely to germinate under moderately high pressure. Indeed, bioinformatics analysis of sequenced *Clostridium* genomes reveals that this is the case for at least the majority of *Clostridium* food-borne pathogens (i.e. *C. botulinum*, *C. perfringens* and *Clostridium difficile*, etc.) (Paredes-Sabja et al., 2011), where the GRs have a different genetic architecture than those found in the most studied *Bacillus* species; therefore, they might have a different conformation and responsiveness to high pressure. In summary, a species-specific experimental approach should be used to develop high pressure strategies that aim to modulate the germination apparatus.

Alternative strategies for high pressure inactivation of bacterial spores: germinants and antimicrobial compounds as hurdle approach

Food safety regulations require that processing regimes target at least an inactivation of 6 DR for the pathogen of interest (Pérez Lamela & Torres, 2008). However, the complexity of the germination machineries of bacterial species of *Bacillus* and *Clostridium* (Paredes-Sabja et al., 2011) makes it difficult to establish a universal two-step high pressure inactivation strategy consisting of a pressure-induced germination step followed by a pressure-inactivation step.

Recent efforts have been made to develop new hurdle technology relying on the synergistic effects of natural antimicrobial compounds with conventional and novel food processing options to reach the target inactivation level (Rastogi et al., 2007). The advantage of these strategies is that they minimize the HHP treatment intensity to commercially feasible levels. The development of successful hurdle strategies depends on an in-depth understanding of their inactivation mechanisms to establish the most effective treatment condition (Gayán et al., 2012).

Two-step HHP-induced spore inactivation process

As mentioned earlier, low- or high-pressure treatments do not trigger the germination of spores of all bacterial species (Paredes-Sabja et al., 2011). Thus, an alternative strategy can be to trigger germination of bacterial spores in the food through the addition of species-specific germinant(s) into the food formulation. In the case of *Bacillus* species, several successful two-step strategies have been developed requiring no germinants. The successful germination and inactivation of *B. cereus* spores (8 DR) in milk has been demonstrated with a single-pressure pulse of 2 min at 650 MPa at 40 °C achieving germination and inactivation levels of spores >5 DR (Raso et al., 1998b). By contrast, germination of spores of the high temperature resistant *Bacillus sporothermodurans* is maximal after a 5-min treatment at 200 MPa and 20 °C (Aouadhi et al., 2012). However, as mentioned earlier, pressure alone is not sufficient to trigger germination of *Clostridium* spores; indicating that additional germinants need to be added to the food formulation to induce germination of their spores. For example, Akhtar et al. (2009) demonstrated that addition of a mixture of 50 mM L-asparagine and KCl to poultry meat formulation triggers germination of *C. perfringens* spores after a heat activation process in heat-treated meats, allowing an efficient inactivation (4 DR) by a subsequent pressure pulse (568 MPa, 75 °C, 15 min). Further research to develop a universal nutrient and/or pressure-induced germination is required to enhance the inactivation efficiency of pressure treatments.

HHP and antimicrobial compounds

In addition to the traditional combination of HHP with mild heat, and germinants, HHP can be combined with bacteriocins (Lee & Kaletunc, 2010) or antimicrobial compounds that exert synergistic effects with HHP (Gayán et al., 2012). Although several studies have suggested that lysozyme can be used to increase the lethality of HHP against vegetative cells (Tribst et al., 2008), work on spores of *B. cereus* have demonstrated that it does not increase the lethality of HHP treatments (Lopez-Pedemonte et al., 2003; Sokolowska et al., 2012). In contrast, the use of bacteriocins has demonstrated that nisin is an effective antimicrobial compound against several bacterial pathogens (Kalchayanand et al., 2003; Sokolowska et al., 2012; Udompijitkul et al., 2012). The combination of 200 MPa for 45 min with a nisin concentration of 250 IU/ml may achieve ~6 DR in the viability of *Alicyclobacillus acidoterrestris* spores in apple juice (Sokolowska et al., 2012). Nisin was also shown to increase the lethality of moderate pressures (550 MPa at 41 °C for 12 min), achieving 6 DR in the viability of *B. coagulans* spores in milk (Gao & Ju, 2011). Similar results were observed when *B. cereus* spores were treated for 5 min with 500 MPa at 40 °C in the presence of 500 IU/ml nisin reaching inactivation levels of 5.8 DR (Black et al., 2008). The presence of nisin also increased the effect of HHP against the spores of *C. perfringens*, with a 654 MPa for 13 min at 74 °C in the presence of ~300 IU/ml, reaching inactivation levels of ~7.8 DR (Gao et al., 2011). These studies demonstrate that the presence of nisin increases the efficiency with which HHP treatments inactivate bacterial spores, however, cautions should be taken to develop cost-effective processing strategies that are commercially feasible.

Besides nisin, several other compounds have been used as antimicrobials in combination with HHP to achieve maximum lethality on bacterial spores and vegetative cells, e.g. lactoperoxidase or lysozyme (Devlieghere et al., 2004). This synergistic effect is advantageous in terms of protection against widespread cellular and biochemical damages due to pressure treatment. For example, lactic acid and sodium sulfate have been exploited for their acidulating property to increase pressure inactivation of bacterial pathogens (Neetoo

et al., 2009). A previous study demonstrated potential synergistic antimicrobial effect with sucrose palmitic acid ester and HPP on *Bacillus stearothermophilus* (Harwood & Cutting, 1990). Similarly, inhibition of *B. subtillus* as a result of HPP in combination with sucrose laurate as antimicrobial has been reported (Stewart et al., 2000).

The use of antimicrobials in combination with HHP provides a good opportunity for food safety improvements, while minimizing the heat treatment required for spore inactivation. Another advantage is the presence of bacteriocin in the finished product offering protection during product storage (Galvez et al., 2010). However, understanding the underlying mechanism on how high levels of inactivation due to combination of antimicrobial agents and HHP hurdles would be achieved, remain to be an important consideration for efficacy of HHP application in the presence of antimicrobials. Primarily, the pressure process entails destabilization of membrane structures and thus increases the cell penetrability by the antimicrobials. Contrarily, treatments with cell-wall weakening agents have been reported to sensitize pressure-resistant bacteria to HHP treatments (Earnshaw et al., 1995). Further research in developing hurdle technology to increase HHP lethality will offer a great variety of solutions by combining novel antimicrobial compounds, germinants, and high-pressure conditions to modulate the germination of bacterial spores and thus achieve their inactivation by milder treatments.

Conclusions and future perspectives

Advances in the understanding of molecular basis of bacterial spore germination and novel food processing technologies have opened new avenues to control spore-forming species. Conventional food pasteurization approaches, primarily based upon high pressure with relatively low temperature, cannot achieve the inactivation of bacterial spores. Thus, an alternative strategy involving induction of bacterial spore germination in the food by addition of specific germinant(s) into the food formulation followed by HHP application has shown promising results. Similarly, using antimicrobials (e.g. lysozyme, nisin and lactoperoxidase) in combination with traditional HHP holds potential to inactivate spores. Further research is warranted towards identifying universal germinants and antimicrobials that can enhance the lethality of HHP treatments on all bacterial spores.

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Declaration of interest

The authors report no conflict of interest.

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