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High hydrostatic pressure-induced inactivation of bacterial spores

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Summary

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 their 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 *Clostridium*. Alternative strategies to inactivate bacterial spores at commercially available pressure and temperature levels include 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.

1. Introduction

New consumer demands for chemical additives reductions and minimally processing of foods have raised the 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 meeting to some extent these consumer demands, while at the same time retaining the sensory and nutritional quality of freshly prepared foods. To date, HHP processing has been the most successful alternative novel technology adopted by the food industry used primarily for the pasteurization of refrigerated low-acid foods.

Industrial HHP processing relies solely on elevated pressure (~400 to 600 MPa) treatments at refrigerated or room temperature (~4 to 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, Valdez-Fragoso, Tonello Samson, Welti-Chanes, & Torres, 2011; Perez Lamela & Torres, 2008; Torres, Sanz, Otero, Pérez Lamela, & Saldaña, 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 the pathogenic and food spoilage bacterial spores levels found in raw foods (E. Gayán, J. A. Torres, & D. Paredes-Sabja, 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 (Perez Lamela & Torres, 2008). Although commercial PATP

prototypes are currently available from Avure Technologies Inc. (www.avure.com) and NC Hyperbaric Inc. (www.hyperbaric.com), 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, Setlow, & Sarker, 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, Paredes-Sabja, Torres, & Sarker, 2009; Black et al., 2008). Bacterial spores lose their inactivation resistance properties upon germination and 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 (E Gayán, J A Torres, & D Paredes-Sabja, 2012). These hurdles to the survival of bacterial spores represent viable strategies for the production of low-acid (pH>4.5) shelf-stable foods relying on HHP treatments. These developments in 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.

2. 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.

2.1. 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, Loessner, & Golden, 2005). HHP treatments at 827 MPa and 75°C barely yield ~3 decimal reductions (DR) in *C. botulinum* spore counts (Reddy, Solomon, Tetzloff, & Rhodehamel, 2003). In addition, *C. botulinum* spore resistance to high pressure in high-acid foods is type-dependent, with type A spores being more sensitive than type B spores (Margosch, Ehrmann, Ganzle, & Vogel, 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, Gonzalez, Sarker, & Torres, 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 leading cause of food poisoning in several countries, and typically associated with low-acid foods with limited refrigerated shelf-life. Similarly 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).

2.2. Food spoilage spore-forming bacterial species. Spore-former species causing food spoilage have major financial consequences in the food industry and 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 their heat resistance of *Bacillus* genus spores, they 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, Cameron, & Witthuhn, 2011). Although spores of *A. acidoterrestis* are slightly heat sensitive ($D_{90°C}$ 15 to 23 min and $D_{95°C}$ 2.4 to

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 (S. Y. Lee, Chung, & Kang, 2006; Silva, Tan, & Farid, 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, and 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, Parris, & Conway, 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, Flint, & Brightwell, 2010), and can grow between -5 to 20°C (Adam, et al., 2010). These species form heat resistant spores that are capable of surviving pasteurization process (Adam, Brunt, Brightwell, Flint, & Peck, 2011) leading to spoilage of meat products typically recognized as “blown pack” or surface spoilage resulting in great economic losses (Adam, et al., 2010). However, there is no available information on their pressure resistance.

3. 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 regulation 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 extended periods of time.

Bacterial spores possess various structural component factors 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 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 possess a lower degree of crosslinking between the glycan strands than in the peptidoglycan cortex of vegetative cells (Popham, Gilmore, & Setlow, 1999), providing the spore cortex with elastic properties that are directly linked to spore wet-heat resistance (Popham, Sengupta, & Setlow, 1995). Below the cortex is a layer of peptidoglycan (PG) cortex with a structure

presumably similar to that of growing cells that becomes the cell wall during spore outgrowh (Setlow, 2003). Beneath the germ cell wall is a compressed spore inner membrane of immobilized lipids (Moir, Corfe, & Behravan, 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, Setlow, Setlow, & Sarker, 2008; 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, Durand, Loison, Perrier-Cornet, & Gervais, 2011; Paredes-Sabja, Sarker, Setlow, Setlow, & Sarker, 2008; Raso, Barbosa-Canovas, & Swanson, 1998). For example, sporulation at temperatures of 42°C yield *C. perfringens* spores that have higher heat resistance than those prepared at 37°C (Paredes-Sabja, Sarker, et al., 2008). Conversely, spores prepared at 27°C had lower resistance than those prepared at 37°C (Paredes-Sabja, Sarker, et al., 2008). Although sporulation conditions variables 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.

4. 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, Zhang, Yu, Setlow, & Li, 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, Setlow, & Setlow, 1981). These later 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, Setlow, et al., 2008). In spores of *Bacillus* species, the release of Ca-DPA activates one of the cortex lytic enzymes (CLEs), CwlJ

(Paidhungat, Ragkousi, & Setlow, 2001), initiating the hydrolysis of the cortex. In parallel the release of material from the spore core produces a decrease in the constraint of the spore PG cortex activating another redundant CLE, SleB (Magge et al., 2008; Tovar-Rojo, Chander, Setlow, & Setlow, 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 stresses. 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.

5. HHP-inactivation strategies for bacterial spores

5.1 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 to 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* (Black, et al., 2005), absence of any single GR does not affect low pressure-triggered spore germination in *B. cereus* (Wei, Setlow, & Hoover, 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, Zhang, Cowan, Yu, & Setlow, 2011) and presumably involved in amplifying the nutrient-induced germination signal that is required for low pressure-induced germination of *B. subtilis* spores (Pelczar, Igarashi, Setlow, & Setlow, 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, Boven, & Michiels, 1998), several differences indicate that low-pressure modulate differently the GRs than nutrient germinants. For example, diacylglycerol covalently added to a cysteine residue in the N-terminal domain of the C 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.

5.2 Moderate pressure-induced germination of *Bacillus* spores. Beside low pressure-induced germination, moderately high pressures (i.e., ~500 to 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, Hirneisen, Hoover, & Setlow, 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 did not (Black, et al., 2007). This suggest 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*.

5.3 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, Chapman, Bull,

293 Heinz, & Knorr, 2007; Mathys, Reineke, Heinz, & Knorr, 2009). The physiological
294 changes of bacterial spores through high pressure are easily quantifiable by flow
295 cytometry and staining with fluorescent SYTO 16 dye (Mathys, et al., 2007). As
296 mentioned above, low and moderate pressure-induced germination not only leads to
297 different spore germination pathways, but also to significant differences in the loss of
298 factors involved in thermal resistance between these two pressure-germination pathways
299 (Coleman, Chen, Li, Cowan, & Setlow, 2007; Setlow, 2007). Both pressure levels trigger
300 the release of Ca-DPA from the spore core and the degradation of the spore PG cortex,
301 which are important factors in thermal resistance. However, a significant difference is
302 that at low but not at moderate pressure-induced germination, *B. subtilis* spores degrade
303 their SASPs, likely via the activation of the germination protease involved in degradation
304 of SASP, GPR (Wuytack, et al., 1998). This might explain why low pressure germinated
305 spores of *B. subtilis* are more easily inactivated than those germinated at 500 MPa, to a
306 subsequent pressure treatment of 600 MPa at 40°C (Wuytack, et al., 1998). This also
307 suggests that SASPs, but not DPA or the integrity of the spore's PG cortex, might play a
308 role in the resistance to pressure with no heating. Despite the lack of degradation of
309 SASPs in *B. subtilis* spores germinated at 500 MPa, these spores were sensitive to UV
310 and hydrogen peroxide treatments (Wuytack, et al., 1998).

311 Modulation of the loss of spore resistance factors can be achieved by changing the
312 pressure-temperature conditions. However, caution should be taken, as most of the
313 enzymes involved in germination might be inactivated after a certain pressure threshold,
314 and this inactivation threshold might be species dependent. For example, it is tempting to
315 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, Mathys, & Knorr, 2011).

5.4 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, Setlow, Driks, & Setlow, 2000; Paredes-Sabja, Setlow, et al., 2008; Reineke, et al., 2011) provide evidence that the mechanism by which spores survive to 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, Setlow, et al., 2008). 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 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 & Shao, 2010; Ramaswamy, Shao, & Songming, 2010) have shown that when pressures higher than 700 MPa are combined with temperatures between 80 to 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.

6. 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 for *Clostridium* spores. However, some very recent studies (Akhtar, et al., 2009; Paredes-Sabja & Sarker, 2010; Paredes-Sabja, Setlow, et al., 2008; Paredes-Sabja, Setlow, & Sarker, 2009a; Paredes-Sabja, Setlow, & Sarker, 2009c, 2009d; Paredes-Sabja, Udompijitkul, & Sarker, 2009) have contributed to significant progress.

362 Most importantly, unlike spores of *Bacillus* species encoding 4 to 8 different ABC
363 tricistronic GR operons, the sequenced genome of *Clostridium* species encodes a lower
364 number of non-tricistronic GRs (Xiao, Francke, Abee, & Wells-Bennik, 2011). Indeed,
365 functional studies in *C. perfringens* SM101 spores have shown that proteins encoded by a
366 bicistronic *gerKA-KC* operon are essential for L-asparagine, Ca-DPA, K^+ and inorganic
367 phosphate as well as for the viability of *C. perfringens* spores (Paredes-Sabja, Setlow, et
368 al., 2009c; Paredes-Sabja, Torres, Setlow, & Sarker, 2008; Paredes-Sabja, Udompijitkul,
369 et al., 2009). In striking contrast to *B. sutbilis* spores, activation of the cortex hydrolysis
370 machinery in *C. perfringens* spores follows a different mechanism that can have major
371 implications in the development of pressure treatments to trigger germination. First, in *C.*
372 *perfringens* spores, Ca-DPA acts through the GerKA-KC receptor pathway and does not
373 directly activate the cortex lytic machinery (i.e., CspB and SleC [CS]) (Paredes-Sabja,
374 Setlow, & Sarker, 2009b; Paredes-Sabja, Setlow, et al., 2009d; Paredes-Sabja, Torres, et
375 al., 2008). Secondly, *C. perfringens* spores that lack DPA are stable and are able to
376 germinate as wild-type spores, indicating that a reduction of the stress of the cortex
377 constrain during DPA release is not required to activate the CS cortex hydrolytic
378 machinery (Paredes-Sabja, Setlow, et al., 2008). The implications of the latter findings
379 are that it is unlikely that moderately high pressures (~500 to 600 MPa) will activate the
380 CS cortex hydrolytic machinery through a DPA-dependent pathway as in *Bacillus* spores
381 (Black, et al., 2007). Thirdly, low pressures (~100 and 150 MPa) do not induce
382 germination in *C. perfringens* SM101 spores within 60 min after pressure treatment
383 (Akhtar, et al., 2009). Similarly, a study using spores of pathogenic *C. perfrigens* 1027
384 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, Dunne, Sikes, & Ray, 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 to 200 MPa) or moderate pressures (~400 – 600 MPa) at either 40 or 60 °C (Mills, Earnshaw, & Patterson, 1998).

When developing pressure induced germination protocols, food processors seek a fast spore germination response after pressure treatments to achieve rapidly a high spore inactivation level (4 to 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, bioinformatic analysis of sequenced *Clostridium* genomes reveals that this is the case for at least the major *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, and 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.

7. 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 (Perez 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, Raghavarao, Balasubramaniam, Niranjan, & Knorr, 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 (E. Gayán, et al., 2012).

7.1 Two-step HHP-induced spore inactivation process. As aforementioned, 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, Gongora-Nieto, Barbosa-Canovas, & Swanson, 1998). 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 above, 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, Ahktar *et al.* (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) (Akhtar, et al., 2009). Further research to develop a universal nutrient and/or pressure induced germination is required to enhance the inactivation efficiency of pressure treatments.

7.2 HHP and antimicrobial compounds. In addition to the traditional combination of HHP with mild heat, and germinants, HHP can be combined with bacteriocins (J. Lee & Kaletunc, 2010) or antimicrobial compounds that exert synergistic effects with HHP (E. Gayán, et al., 2012). Although several studies have suggested that lysozyme can be used to increase the lethality of HHP against vegetative cells (Tribst, Franchi, & Cristianini, 2008), work with spores of *B. cereus* have demonstrated that it does not increase the lethality of HHP treatments (Lopez-Pedemonte, Roig-Sagues, Trujillo, Capellas, &

454 Guamis, 2003; Sokolowska et al., 2012). In contrast, the use of bacteriocins has
455 demonstrated that nisin is an effective antimicrobial compounds against several bacterial
456 pathogens (Kalchayanand, Dunne, Sikes, & Ray, 2003; Sokolowska, et al., 2012;
457 Udompijitkul, Paredes-Sabja, & Sarker, 2012). The combination of 200 MPa for 45 min
458 with a nisin concentration of 250 IU/ml may achieve ~6 DR in the viability of
459 *Alicyclobacillus acidoterrestris* spores in apple juice (Sokolowska, et al., 2012). Nisin
460 was also shown to increase the lethality of moderate pressures (550 MPa at 41°C for 12
461 min), achieving 6 DR in the viability of *B. coagulans* spores in milk (Y. L. Gao & Ju,
462 2011). Similar results were observed when *B. cereus* spores were treated for 5 min with
463 500 MPa at 40°C in the presence of 500 IU/ml nisin reaching inactivation levels of 5.8
464 DR (Black, et al., 2008). Presence of nisin also increased the effect of HHP against
465 spores of *C. perfringens*, with a 654 MPa for 13 min at 74°C in the presence of ~300
466 IU/ml, reaching inactivation levels of ~7.8 DR (Y. Gao, Qiu, Wu, & Fu, 2011). These
467 studies demonstrate that the presence of nisin increases the efficiency with which HHP
468 treatments inactivate bacterial spores, however cautions should be placed to develop cost
469 effective processing strategies that are commercially feasible.

470 Besides nisin, several other compounds have been used as antimicrobials in
471 combination with HHP to achieve maximum lethality on bacterial spores and vegetative
472 cells e.g. lactoperoxidase or lysozyme (Devlieghere, Vermeiren, & Debevere, 2004). This
473 synergistic effect is advantageous in terms of protection against widespread cellular and
474 biochemical damages due to pressure treatment. For example, lactic acid and sodium
475 sulfate have been exploited for their acidulating property to bring about pressure
476 inactivation of bacterial pathogens (Neetoo, Ye, & Chen, 2009). A previous study

demonstrated potential synergistic antimicrobial effect with sucrose palmitic acid ester and HPP on *Bacillus stearothermophilus* (Harwood, 1990). Similarly, inhibition of *B. subtillus* as a result of HPP in combination with sucrose laurate as antimicrobial has been reported (Stewart, Dunne, Sikes, & Hoover, 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, Abriouel, Benomar, & Lucas, 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 increasing 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, Appleyard, & Hurst, 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.

8. 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 spore. 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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