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Microbiological food safety assessment of high hydrostatic pressure processing: A review

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ABSTRACT

High hydrostatic pressure (HHP) processing as a novel non-thermal method has shown great potential in producing microbiologically safer products while maintaining the natural characteristics of the food items. Scientific research of the process and its industrial applications has been widespread in the past two decades with many scientific publications describing its uses, advantages and limitations. The review describes the effect of HHP on foodborne pathogenic microorganisms, their structures and adaptive mechanisms, the intrinsic and extrinsic factors that affect its application with a focus on microbiological safety, and research needs. In a risk assessment context, tools and mechanisms in place to monitorize, optimize and validate the process, and procedures for assessing and modelling the lethal effect of the treatment are reviewed.

1. Introduction

The application of high hydrostatic pressure (HHP) in food preservation has received particular attention as a viable alternative (economically and technologically) to thermal processes (Patterson, 2005). The first research on the effect of high pressure on food was first carried out in the nineteenth century (Hite, 1899) describing an increase in shelf-life for products such as milk, fruit and other foods, but the scientific development, its application in the food industry, and the foodstuff marketing are much more recent and have taken place in the past two decades (Considine, Kelly, Fitzgerald, Hill, & Sleator, 2008; Devlieghere, Vermeiren, & Debevere, 2004; Farr, 1990). At present, thanks to technological improvements in equipment, industrial application is widespread for a range of pressures between 100 and 800 MPa, depending on the desired objective. The process is isostatic, i.e. the pressure is transmitted uniformly and instantly, and adiabatic, which means that no matter the food shape or size, there is little variation in temperature with increasing pressure (the temperature increases approximately 3 °C per 100 MPa, depending on the composition of the food) (Smelt, 1998; Wilson, Dabrowski, Stringer, Moezelaar, & Brocklehurst, 2008). This prevents the food from being deformed or heated, which would modify its organoleptic properties.

One of the principal advantages of the HHP process is the expanded shelf-life and improvement of food safety due to the inactivation affected in the microbial population. The loss of viability of microorganisms through HHP is probably the result of a combination of injuries in the cell. The resistance of microorganisms is highly variable, depending mainly on the type of organism and the food matrix involved, e.g. spores show great resistance to inactivation. The introduction of an HHP step in the food manufacturing process requires a careful assessment of microbiological risks. The process safety assurance is enhanced when an adequate hazard identification is performed. As with other treatments, there is also a need to have tools and mechanisms in place to monitor, optimize and validate the process, and procedures to assess and model the lethal effect of the treatment.

This article will review the updated knowledge dealing with the effect of HHP on foodborne pathogenic microorganisms and their structures, and the adaptive mechanisms, the intrinsic and extrinsic factors that affect its application, and research needs. Special attention is paid to the identification and evaluation of microbiological hazards as well as the monitorization, optimization and validation of HHP treatments, and methodologies for assessing and modelling the lethal effect of HHP processes.

2. Effect of HHP on molecules and structures

High hydrostatic pressure does not alter the low-energy, covalent bonds, which have low compressibility and do not break within the ranges of pressures normally used in food. Therefore

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the primary structure of molecules such as proteins or fatty acids remains intact (Considine et al., 2008). The ionic bonds and hydrophobic interactions, responsible for maintaining the secondary and tertiary structure of proteins, are disrupted and this event is associated with decreases in volume (Aymerich, Picouet, & Monfort, 2008; Considine et al., 2008; Heremans, 1995; Ross, Griffiths, Mittal, & Deeth, 2003). The secondary, tertiary or quaternary structure of large molecules and complex organized structures such as membranes are altered, due to the rupture of ionic bonds, some hydrogen bonds and hydrophobic and electrostatic interactions. Large macromolecules (starch) undergo changes such as gelling. The global consequences for the product are diverse, since the activity of certain enzymes can be inhibited, the nutrient digestibility and bioavailability modified, or the technological and functional properties altered. Molecules such as vitamins, amino acids, flavour molecules or other low-molecular-weight compounds are hardly affected and as a result, the organoleptic and nutritional properties are slightly modified (Aymerich et al., 2008; Farkas & Hoover, 2000). Applications of HHP in food technology deal with changes in the functional capacity and structure of proteins (Huppertz, Smiddy, Upadhyay, & Kelly, 2006; San Martin, Barbosa-Canovas, & Swanson, 2002) changes in the enzyme activity (Lopez-Malo, Palou, Barbosa-Canovas, Welti-Chanes, & Swanson, 1998), heat transfer (Otero & Sanz, 2003), combined dehydration processes (Ade-Omowaye, Rastogi, Angersbach, & Knorr, 2001), and compound extraction systems (Jun, 2006), among others.

Apparently, there is no single damage in a cellular structure or function (Hoover, Metrick, Papineau, Farkas, & Knorr, 1989; Manas & Mackey, 2004; Ritz, Tholozan, Federighi, & Pilet, 2001). Cell death is due to a multiplicity of damage accumulated in different parts of the cell (Malone, Shellhammer, & Courtney, 2002). When the accumulated damage exceeds the cell's ability to repair, death occurs. On occasions, the cell is damaged but could recover if post-treatment conditions are appropriate (Bozoglu, Alpas, & Kaletunc, 2004; Bull, Hayman, Stewart, Szabo, & Knabel, 2005). Because of its special features, the cell membrane is the main target of HHP (Pagan & Mackey, 2000; Ritz, Freulet, Orange, & Federighi, 2000; Ritz, Tholozan, Federighi, & Pilet, 2002; Russell, 2002; Smelt, 1998), resulting mainly in permeability modification and functionality disruption (Pagan & Mackey, 2000). Leaking has been shown since intracellular fluid compounds have been detected in the cell-suspending liquid after HHP processing (Shimada et al., 1993).

The different chemical composition and structural properties of the cell membrane in Gram-positive and Gram-negative microorganisms result in differences in resistance to HHP (Russell, 2002). Gram-positive bacteria are generally more resistant compared to Gram-negative (Shigehisa, Ohmori, Saito, Taji, & Hayashi, 1991). Apparently, the double-layered phospholipids which are present in the lipid membranes are packed tightly during the compression phase, promoting the transition towards a gel state (Hazel & Williams, 1990), and during decompression the dual layer structure is lost, with pore formation and cytoplasmic material leaking (Hoover et al., 1989; Shimada et al., 1993). The membrane must maintain the fluid state to maintain its function and properties, and fluidity is mainly determined by composition and percentages of unsaturated fatty acids (FA). The FA in the membrane of barophiles and barotolerants have a greater degree of unsaturation (Smelt, 1998; Yano, Nakayama, Ishihara, & Saito, 1998). It has also been observed that psychrophiles, adapted to grow at low temperatures and containing high levels of polyunsaturated FA (docosahexaenoic acid), are generally more pressure-resistant, confirming that fluid membranes, rich in unsaturated FA, are partially responsible for resistance to HHP lethal effects (Casadei, Manas, Niven, Needs, & Mackey, 2002; Smelt, 1998). Adaptive changes in response to increases in pressure, such as the shift from saturated to unsaturated FA, have been described (Yano et al., 1998). The inactivation of the ATPase of the cell membrane or other proteins such as efflux pumps also affects membrane function and alters the acid–base physiology of the cell (Hoover et al., 1989; Russell, 2002). Modifications in the cell membrane of *Salmonella* Typhimurium after HHP treatments (600 MPa) are visible by using SDS-PAGE electrophoresis, with disappearance of most outer membrane proteins, while major proteins OmpA, OmpC and LamB are baroresistant (Ritz et al., 2000). There are other components and cellular functions sensitive to high pressures that are altered or inhibited such as the ribosome (Kaletunc, Lee, Alpas, & Bozoglu, 2004), the protein synthesis and enzyme activity (Considine et al., 2008; Simpson & Gilmour, 1997; Wouters, Glaasker, & Smelt, 1998) and the structure of DNA-enzyme complexes, as the DNA can be degraded due to the action of endonucleases not normally in contact with DNA (Chilton, Isaacs, Mackey, & Stenning, 1997).

A series of morphological and structural changes in the cell, such as the separation of the membrane from the cell wall, the lengthening of the cell, the compression of gas vacuoles (Patterson, 2005) and the condensation of nuclear material (Manas & Mackey, 2004; Wouters et al., 1998), are also described. In addition, HHP treatments are reported to have induced a series of changes in the cell, replying in a similar manner to when exposed to other stressors: SOS response, cold and heat stress and oxidative stress response, changes in the genetic regulation of quimiotaxis gene, phosphotransferase production, flagella and expression of genes involved in cell elongation and development of septa (Aertsen, Van Houdt, Vanoirbeek, & Michiels, 2004; Aertsen, De Spiegeleer, Vanoirbeek, Lavilla, & Michiels, 2005; Aertsen, Vanoirbeek, et al., 2004; Malone, Chung, & Yousef, 2006).

The exact mechanisms of spore inactivation are not known but it is hypothesized that spores first are activated due to particular pressure/temperature conditions, losing their inherent resistance, and are subsequently killed by treatment conditions. This mechanism is used advantageously in HHP processing. During inactivation a series of events such as the release of dipicolinic acid (chelate of Ca^{2+} and pyridine-2,6-dipicolinic acid or Ca-DPA) and small acid-soluble spore proteins (SASPs), the hydrolysis of core and cortex, and the decrease of intracellular pH occur (Ahn & Balasubramaniam, 2007b). Spores are sensitized by moderately high pressures (50–300 MPa), and it has been postulated that nutrient–germinant receptors are activated by low-level HHP, which in turn, provokes the release of Ca-DPA. Ca-DPA triggers a cascade of later germination events (hydrolysis of spore cortex by cortex-lytic enzymes (CLE), degradation of SASPs and ATP generation) (Paidhungat et al., 2002). The resultant germinated spores are sensitive to subsequent heat and pressure treatments (Black et al., 2005; Black, Setlow, et al., 2007). Also, high pressures (>500 MPa) can induce rapid germination by direct release of Ca-DPA (Wuytack, Boven, & Michiels, 1998).

3. Effect of HHP on the microbiota

As a food preservation technology, the utility of HHP is due to the destruction suffered by the microbial population, which allows a substantial increase in shelf-life and improves food safety (Considine et al., 2008). Broadly speaking, HHP applied at ambient temperature destroys vegetative cells and inactivates certain enzymes (Simpson & Gilmour, 1997), with a minimal change in the organoleptic properties (Farkas & Hoover, 2000; San Martin et al., 2002). The effectiveness of the treatment depends primarily on the pressure applied and on the holding time. The resistance of microorganisms is highly variable, depending mainly on the type

Table 1
Viability loss of vegetative pathogens and foodborne viruses by HHP with different time, temperature and pressure combinations.

	Substrate	P (MPa)	Time (min)	T (°C)	Inactivation	Reference
Campylobacter jejuni	Pork slurry	300 MPa	10 min	25 °C	6 log CFU	(Shigehisa et al., 1991)
Salmonella Senftenberg 775W	Strained baby food	340 MPa	10 min	23 °C	<2 log CFU	(Metrick, Hoover, & Farkas, 1989)
Salmonella Enteritidis	Broth	345 MPa	10 min	35 °C	8,22 log CFU	(Alpas et al., 2000)
Escherichia coli O157:H7	Poultry meat	600 MPa	15 min	20 °C	3 log CFU	(Patterson et al., 1995)
E. coli O157:H7	Broth	345 MPa	10 min	35 °C	8.14 log CFU	(Alpas et al., 2000)
Staphylococcus aureus	Poultry meat	600 MPa	15 min	20 °C	3 log CFU	(Patterson et al., 1995)
S. aureus 765	Broth	345 MPa	10 min	35 °C	4 log CFU	(Alpas et al., 2000)
Listeria monocytogenes CA	Poultry meat	375 MPa	15 min	20 °C	2 log CFU	(Patterson et al., 1995)
L. monocytogenes	Broth	345 MPa	10 min	35 °C	5 log CFU	(Alpas et al., 2000)
Vibrio parahaemolyticus O3:K6	Oysters	300 MPa	3 min	10 °C	5 log CFU	(Cook, 2003)
Hepatitis A virus	Oysters	400 MPa	1 min	10 °C	>3 log PFU	(Calci et al., 2005)
Norovirus	Oysters	400 MPa	5 min	5 °C	4 log PFU	(Kingsley et al., 2007)

of organism and the food matrix, as discussed below. As previously noted, pressure can also impair sublethal injury to the cell.

High hydrostatic pressure usually has a higher destructive effect in organisms with a greater degree of organization and structural complexity. Prokaryotes are usually more resistant, compared to eukaryotes (Yuste, Capellas, Fung, & Mor-Mur, 2001). The destruction of protozoa and parasites is achieved with relatively low pressures. Studies on the effectiveness of HHP in eliminating foodborne parasites show the sensitivity of protozoa and parasites (*Toxoplasma gondii* (Lindsay, Collins, Holliman, Flick, & Dubey, 2006), *Cryptosporidium parvum* (Collins et al., 2005), *Anisakis simplex* (Brutti et al., 2010; Molina-Garcia & Sanz, 2002), *Trichinella spiralis* (Noeckler, Heinz, Lemkau, & Knorr, 2001), *Ascaris* (Rosypal, Bowman, Holliman, Flick, & Lindsay, 2007)) in low pressure ranges (100–400 MPa). Moulds and yeasts have intermediate resistance (Palou et al., 1998b; Palou, Lopez-Malo, Barbosa-Canovas, Welti-Chanes, & Swanson, 1997; Shimoda et al., 2002). As for moulds, mycelia are particularly susceptible, but mould spores particularly resistant to high pressures have been reported (*Neosartorya, Talaromyces* (Chapman et al., 2007; Smelt, 1998)). The ascosporas of *Byssochlamys* spp. can withstand high pressures (689 MPa) for extended periods and an increase in processing temperature is needed to obtain substantial loss of viability (Palou et al., 1998a). The experiments carried out on *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* show that their susceptibility is high and they behave similarly to bacteria exhibiting a large influence of environmental factors (Palou et al., 1998b, 1997).

In studies with foodborne vegetative pathogens (Alpas et al., 1999), large viability losses (reductions in excess of 8 logs) are shown when relatively low pressures (300 MPa) are combined with intermediate temperatures (50 °C, 5 min). Large differences among pathogenic microorganisms (*Listeria monocytogenes, Staphylococcus aureus, Escherichia coli, Salmonella* Typhimurium) have been reported, with reductions in the range of 0.5–8.5 log units (Table 1). Also, differences between pathogenic strains belonging to the same genus or species have been described (Alpas et al., 1999; Benito, Ventoura, Casadei, Robinson, & Mackey, 1999; Bull, Olivier, van Diepenbeek, Kormelink, & Chapman, 2009; Garcia-Graells, Valckx, & Michiels, 2000).

Spores show great resistance to inactivation by high pressure. The genera *Bacillus* and *Clostridium* comprise significant species as foodborne sporeforming pathogens, such as *Clostridium botulinum*, *Clostridium perfringens* and *Bacillus cereus*. To aid in the calculation of process specifications, suitable surrogates of *C. botulinum* such as *Clostridium sporogenes* have been investigated (Ahn, Balasubramaniam, & Yousef, 2007). As for other species, *Bacillus amyloliquefaciens* forms extremely high-pressure resistant spores and it has been suggested that it could be adopted as the target organism in the development of standards for high-pressure treatments (Margosch, Ehrmann, Ganzle, & Vogel, 2004; Rajan, Ahn, Balasubramaniam, & Yousef, 2006). Spores of this species in a carrot pulp matrix withstood 16 min at 800 MPa and 70 °C without reduction of spore counts (Margosch, Ehrmann, et al., 2004). Endospores of *C. botulinum* are also extremely resistant to HHP with variations among types (Table 2) (Margosch et al., 2006; Margosch, Ehrmann, et al., 2004; Reddy et al., 1999; Reddy, Solomon, Tetzloff, & Rhodehamel, 2003; Reddy, Tetzloff, Solomon, & Larkin, 2006). There are few results on the behavior of *B. cereus* in foods (Oh & Moon, 2003; Van Opstal, Bagamboula, Vanmuysen, Wuytack, & Michiels, 2004) but in all cases a combination of high pressure (>600 MPa) and mild temperature (>45 °C) had to be used to achieve a significant loss of viability. In the studies involving *C. perfringens* (Papafragkou, Hoover, & Daniels, 2002; Paredes-Sabja, Gonzalez, Sarker, & Torres, 2007) there was modest or no reduction in spore counts for the conditions tested (Table 2).

Viruses possess a wide range of pressure resistance, depending on their structural diversity. Enveloped viruses are usually more sensitive to the pressure than naked viruses. Reduced time–pressure combinations are effective in decreasing the infective power of hepatitis A virus (Calci, Meade, Tezloff, & Kingsley, 2005;

Table 2
Viability loss of foodborne pathogenic spores by HHP with different time, temperature and pressure combinations.

	P (MPa)	Time (min)	T (°C)	Inactivation	Reference
B. cereus	400	25	30	0.5 log	(McClemens, Patterson, & Linton, 2001)
B. cereus	600		60	6–7log	(Oh & Moon, 2003)
C. perfringens	500	30	25, 45 and 65	Minimal or no reduction	(Papafragkou et al., 2002)
C. perfringens type A	650	15	75	3.7 log	(Paredes-Sabja et al., 2007)
C. botulinum type E	827	5	50–55	Approx. 5 log	(Reddy et al., 1999)
C. botulinum nonproteolytic type B	827	20	75	> 6 log	(Reddy et al., 2006)
C. botulinum type A	827	20	75	2-3 log	(Reddy et al., 2003)
C. botulinum proteolytic Type B	600	70	80	5 log	(Margosch, Ehrmann, et al., 2004; Margosch, Ganzle, et al., 2004)
C. botulinum proteolytic Type B	800	4	80	2.3 log	(Margosch, Ehrmann, et al., 2004; Margosch, Ganzle, et al., 2004)
C. botulinum proteolytic Type A	600	6	80	5 log	(Margosch, Ehrmann, et al., 2004; Margosch, Ganzle, et al., 2004)

Kingsley, Guan, Hoover, & Chen, 2006) and norovirus (Buckow, Isbarn, Knorr, Heinz, & Lehmacher, 2008; Kingsley, Holliman, Calci, Chen, & Flick, 2007) (Table 1). The polioviruses seem to be the most resistant virus. HHP denatures capsid proteins in a reversible or irreversible way, depending on the pressure. The inactivation rate for viruses follows generally non-linear kinetics, fitting Weibull or log-logistic models (Buckow et al., 2008). Usually, better rates of inactivation are observed at refrigeration temperatures (except for the hepatitis A virus). Important extrinsic factors that affect the efficacy of HPP are the ionic strength of the food matrix and temperature (Baert, Debevere, & Uyttendaele, 2009; Calci et al., 2005). Also for viruses HHP resistance varies within related taxonomic groups or even strains, and differences in protein sequence and structure are responsible (Baert et al., 2009).

To summarize, for most forms of vegetative bacteria, significant reductions (usually higher than 4 log units) in the population are achieved when 400–600 MPa at room temperature are used. However, under these conditions, significant reductions in the load of spores are not achieved. To this aim, other strategies have been assayed (very high pressures, combined use of temperatures and pressure, pressure pulsing) (Ahn et al., 2007; Bari, Ukuku, Mori, Kawamoto, & Yamamoto, 2008; Black, Huppertz, Kelly, & Fitzgerald, 2007; Black, Setlow, et al., 2007; Margosch, Ehrmann, et al., 2004; Rajan, Ahn, et al., 2006; Ratphitagsanti, Ahn, Balasubramaniam, & Yousef, 2009; Smelt, 1998; Wilson et al., 2008).

4. Intrinsic and extrinsic factors that affect pressure resistance

Experiments in model systems show that the physiological status of microbial populations subjected to HHP processing influence pressure resistance. Bacteria are more susceptible during the logarithmic phase as compared to stationary phase, similar to what occurs with other technological processes such as heating (Hayman, Anantheswaran, & Knabel, 2007; Linton, McClemens, & Patterson, 2001; Manas & Mackey, 2004; Pagan & Mackey, 2000). Previous incubation temperatures of microorganisms is also important (Bull et al., 2005). In general, the synthesis of proteins that protect against adverse conditions (like high concentrations of salt, acid conditions, high and low temperatures, oxidative stress) increases resistance to HHP (Considine et al., 2008; Wemekamp-Kamphuis, Karatzas, Wouters, & Abee, 2002; Wemekamp-Kamphuis et al., 2004). Membrane composition, stationary phase proteins and stress proteins are reported to affect pressure resistance in *L. monocytogenes* (Hayman et al., 2007). Prior heat shock increases the barotolerance of *L. monocytogenes* due to *de novo* protein synthesis during heat shock (Hayman, Anantheswaran, & Knabel, 2008). If sporulation takes place at lower temperatures (<30 °C) the pressure resistance of spores increases (Igura, Kamimura, Islam, Shimoda, & Hayakawa, 2003). In foods, microbial populations are diverse and in very different physiological states. Exposure to cold temperatures before processing increases the percentage of polyunsaturated FA in cell membranes, and therefore the resistance to HHP processing. On the contrary, cells already supporting a sublethal injury (e.g., due to thermal treatments, freezing processes or irradiation) become more susceptible to pressure.

The microbial susceptibility to HHP inactivation is clearly influenced by the conditions of the environment where microorganisms are present. Therefore, the results obtained in model systems using artificial substrates cannot be directly compared to "real" foods, and should be validated (Claeys, Van Loey, & Hendrickx, 2003). The chemical composition of the food is important, since the presence of fats, proteins, minerals and sugars serves as a protector and increases microbial resistance to pressure (Black, Huppertz, et al., 2007; Hauben, Bernaerts, & Michiels, 1998; Molina-Hoppner, Doster, Vogel, & Ganzle, 2004). Nutrient-rich foods, such as milk or poultry meat, can protect microorganisms (Patterson, Quinn, Simpson, & Gilmour, 1995).

The temperature of the product and pressure fluid can affect microbial resistance, with larger inactivation rates obtained above or below the ambient temperature. Therefore, process efficacy is influenced by the processing temperature (Alpas, Kalchayanand, Bozoglu, & Ray, 2000; Bayindirli, Alpas, Bozoglu, & Hizal, 2006). The decrease in resistance to pressure at low temperatures may be due to changes in membrane structure and fluidity through weakening of hydrophobic interactions and crystallisation of phospholipids (Cheftel, 1995). Moderate heating (40–60 °C) can also enhance microbial inactivation by pressure, which in some cases make application of lower pressure an option (Carlez, Rosec, Richard, & Cheftel, 1993). Susceptibility to pressure increases visibly as pH deviates from neutral values (Alpas et al., 2000). HHP may inactivate membrane proteins responsible for regulating the trans-membranous flow of protons (efflux pumps), leading to inability to maintain the homeostasis (Hoover et al., 1989). A previous adaptation to environmental conditions can modify susceptibility, and cells of *Lactobacillus plantarum* grown at pH 5.0 were more resistant to pressures of 250 MPa than were cells grown at pH 7.0 (Wouters et al., 1998). There is theoretical evidence that, during pressurization, a temporary reduction of pH and an increase in the dissociated form of the organic acids could be present (Palou, Lopez-Malo, Barbosa-Canovas, & Swanson, 2007). Carboxylic acids normally employed as food preservatives have a high degree of dissociation under conditions of high pressure, and it is the non-dissociated form which has an antimicrobial effect. Lower values of water activity (a_w) increase microbial resistance (Black, Huppertz, et al., 2007; Black, Setlow, et al., 2007; Hayman, Kouassi, et al., 2008; Patterson, 2005). This phenomenon is observed both in synthetic models and food. The efficacy of HHP processing decreases with reduced a_w, and it is visibly observed in foods with values below 0.9. This fact also applies to spores such as those from *B. cereus* (Raso, Gongora-Nieto, Barbosa-Canovas, & Swanson, 1998) and can be attributed, at least partially, to incomplete germination in conditions of low water availability (Black, Huppertz, et al., 2007; Black, Setlow, et al., 2007). Ionic solutes such as NaCl or $CaCl_2$ offer more protection to *Bacillus coagulans*, compared with non-ionic solutes such as sucrose and glycerol (Patterson, 1999). Variations in pressurization equipment can yield different microbial survival results during storage, and different pressure–time profiles (including come-up time and depressurization) at different locations in the vessel may result in a heterogeneous distribution of microbial inactivation within the product (Hugas, Garriga, & Monfort, 2002).

The combined use of antimicrobial compounds (bacteriocins, lysozyme, chitosan, lactoperoxidase system, essential oils) together with HHP has been tested in searching for synergistic effects (Garriga, Aymerich, Costa, Monfort, & Hugas, 2002; Hugas et al., 2002; Kalchayanand, Sikes, Dunne, & Ray, 1998; Kalchayanand, Sikes, Dunne, & Ray, 1994; Karatzas, Kets, Smid, & Bennik, 2001; Rodriguez, Arques, Nunez, Gaya, & Medina, 2005). There are many bacteriocin-producing lactic acid bacteria that can be found naturally in food or intentionally added as starter cultures in fermented foods. Nisin and lysozyme have been commonly used in combination with HHP (Kalchayanand et al., 2004; Masschalck, Van Houdt, Van Haver, & Michiels, 2001). Nisin is a peptide with activity at the cytoplasmic membrane, forming pores that affect stability. Lysozyme targets the peptidoglycan layer, hydrolyzing glycosidic links between saccharides (N-acetyl muramic acid and N-acetyl-D-glucosamine). Under normal conditions its activity is restricted

to Gram-positive bacteria, whose cell wall is not protected by lipopolysaccharides normally present in the outer cell membrane of Gram-negative bacteria. It can also be active in Gram-negative bacteria when the barrier properties of the membrane are removed (Kalchayanand et al., 1998; Masschalck et al., 2001). Another system used in combination with HHP is the lactoperoxidase (Garcia-Graells et al., 2000).

After processing, survivors behave in a different way, showing variations in the kinetic growth parameters (lag phase and growth rate) (Lakshmanan & Dalgaard, 2004). This phenomenon can be explained by the variable composition in FA present in the membrane and other structures, and must be taken into account when evaluating HHP treatments; to mitigate this effect cocktails of strains are frequently used in models (Bull et al., 2005). The availability of some substrates or the presence of factors in the matrix such as vitamins and amino acids in the food (or the growth medium) allows better recovery of sublethally damaged cells after processing (Tassou, Galiatsatou, Samaras, & Mallidis, 2007). The kind of solute (salt or sugar) can have a significant influence on cell survival after processing, and especially on the resistance of the spores (Patterson, 2005).

All these data indicate that, when planning an HHP treatment to ensure the microbiological stability of food and its safety, it is important to consider the utilization of fractional factor designs as a reliable and cost effective methodology in experiment planning. The synergistic effect of pre- and post-processing factors ("hurdle" effect) should be explored, since microbial inactivation rates can be further enhanced, as compared to HHP processing alone.

5. Assessment to determine the microbiological safety of HHP

High-pressure-treated foodstuffs could be considered "novel foods", and in accordance related issues are covered by Regulation (EC) No 258/97,[1] which regards as novel foods those that do not have a significant history of consumption within the European Union before 15th May 1997, and, regarding processing aspects, foods and food ingredients to which a production process not currently used has been applied, where that process gives rise to significant changes in the composition or structure of the foods or food ingredients, affecting their nutritional value, metabolism or level of undesirable substances. In case a food is considered as novel, applications for authorization issued by the food company are received by the corresponding Member State (MS) in which the food is marketed, which carry out the (pre-market) scientific assessment of the safety of the product (Heinz & Buckow, 2010). The rest of the MS can also object to the authorization procedure. Not only microbiological considerations but also toxicological, nutritional, intended use and allergenic aspects should be also part of a risk evaluation process. Several products, such as high-pressure-pasteurized orange juice (France), cooked ham (Spain), oysters (Great Britain), fruits (Germany) have been evaluated at national level with a common agreement that the high pressure treatment has not caused significant changes in the composition or the structure of the products affecting their nutritional value, metabolism or the amounts of undesirable substances (Eisenbrand, 2005). The general aspects of Risk Assessment applied to HHP-processed foods have been partly covered by some Risk evaluation agencies (German Senate Commission on Food Safety (SKLM) of the German Research Foundation (DFG); (Eisenbrand, 2005)) and the AESAN (Agencia Española de Seguridad Alimentaria, 2003).

[1] Regulation (EC) No 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients.

5.1. Identification of microbiological hazards associated with HHP

In each food a list of potential hazards originating from the raw materials or introduced during food handling and processing can be identified. *Listeria monocytogenes* is the logical target in the production of sliced ready-to-eat (RTE) meat products. In oysters meant to be eaten raw pathogenic *Vibrio* are among the microorganisms identified as hazards. For low-acid, shelf-stable foods, *C. botulinum* is the most important microbiological hazard and the critical target in the calculation of process conditions. Extreme baroresistant spoilage microorganisms such as sporeforming bacteria (*Bacillus amyloliquefaciens, Thermoanaerobacterium thermosaccharolyticum*) can also be identified as targets in case a pressure-assisted thermal sterilization treatment is required(Ahn et al., 2007; Margosch, Ganzle, Ehrmann, & Vogel, 2004; Rajan, Ahn, et al., 2006). Other essential factors are the production stage where HHP is applied and the needs of food processors, which are diverse (e.g. production of pathogen-free foods, such as RTE; inactivation of spores to obtain a shelf-stable product; pasteurization of foods with high microbial load).

The introduction of an HHP process in the food industry requires a careful assessment and identification of microbiological hazards. This is especially important in the early stages of process implementation on an industrial scale. The assessment should consider the prevalence and level of potential biological hazards present in the raw materials or introduced during food handling and processing, intrinsic and extrinsic conditions during processing which have an important influence on HHP inactivation (see above), and factors before and after processing. The safety assurance is improved when in addition to a proper risk evaluation, procedures for assessing the lethal effect of the treatment are included as well as mechanisms to monitor, evaluate, optimize and validate the lethal burden of the process. Especially if the assessments are performed in model systems, validation of mathematical models in foods is necessary, together with challenge tests and shelf-life tests (Aymerich et al., 2008; Bozoglu et al., 2004).

The possible selection of microorganisms resistant to pressure after successive treatments is a controversial issue in relation to HHP processing. An undesirable consequence would be the introduction of resistant strains in the environment of the food industry. Both spoilage and pathogenic microorganisms with marked characteristics of tolerance to pressure have been described. Microorganisms of the species *E. coli* resistant to pressure (800 MPa) have been isolated, by applying cycles of exposure in the range 280–450 MPa (Hauben et al., 1997). Strains of *Listeria monocytogenes* that have resisted processing conditions of 400 MPa for 20 min have also been characterized (Karatzas & Bennik, 2002). This fact has also been described for viruses (Smiddy, Kelly, Patterson, & Hill, 2006). The existence of viable non-culturable forms (*Vibrio* spp.) or cells in a dormant, long-term-survival phase (*L. monocytogenes*), with a higher resistance when compared with vegetative cells of the same species has also been described (Berlin, Herson, Hicks, & Hoover, 1999; Wen, Anantheswaran, & Knabel, 2009). The presence of cold-shock membrane proteins, either present or induced *per se* prior to treatment, can increase resistance to pressure (Wemekamp-Kamphuis et al., 2002). The activation of certain genes (producers of the *RpoS* protein in *E. coli* and *SigB* in *L. monocytogenes*) directly affect the degree of resistance to pressure (Malone et al., 2006; Robey et al., 2001; Wemekamp-Kamphuis et al., 2004).

The production of shelf-stable, low-acid foods requires the inactivation of spores by combining HHP with other process, usually elevated temperature(Black, Huppertz, et al., 2007; Black, Setlow, et al., 2007; Wilson et al., 2008). At standard pressures used in food processing, spores are not inactivated and a pasteurized

product is obtained, in which surviving spores have no competitors. In these conditions, the risks posed by pathogenic spores should not be underestimated. Procedures that achieve significant reductions in the spore count have been described. One strategy is to induce its germination using pressure (Black et al., 2005; Black, Setlow, et al., 2007; Paidhungat et al., 2002; Smelt, 1998; Wuytack et al., 1998) or mild heating processes. Pressure pulsing has been successfully tested(Ahn & Balasubramaniam, 2007a; Bari et al., 2008; Lopez-Pedemonte, Roig-Sagues, Trujillo, Capellas, & Guamis, 2003; Ratphitagsanti et al., 2009). The spores germinated can be destroyed later in a new cycle using higher pressures. Another system would be through the combined, simultaneous use of HHP and mild heating (Clery-Barraud, Gaubert, Masson, & Vidal, 2004; Lee, Dougherty, & Kang, 2002; Margosch et al., 2006; Margosch, Ganzle, et al., 2004; Patazca, Koutchma, & Ramaswamy, 2006). The use of combined cycles seems to have particular relevance to the inactivation of spores (Ahn et al., 2007; Heindl et al., 2008; Shen, Urrutia Benet, Brul, & Knorr, 2005). Nonetheless, these methods cannot yet reliably be adopted commercially for food sterilization without a proper assessment of the effectiveness against pressure-resistant pathogenic spores, due to the variable effects of HHP on spore germination (Ahn et al., 2007; Wilson et al., 2008). It should be indicated that pressure resistance of spores is not usually correlated to heat resistance (Margosch, Ehrmann, et al., 2004; Patazca et al., 2006).

There are particular areas where knowledge gaps have been identified. Additional data on the inactivation of bacterial toxins by HHP is needed (Eisenbrand, 2005; Margosch et al., 2005; Murchie et al., 2005). Attention should be given to the presence in raw materials of low molecular weight molecules, such as biotoxins, which, due to their lack of secondary, tertiary and quaternary structure, could be HHP resistant and remain in the product after processing, as it happens with other treatments such as the heat treatment (Murchie et al., 2005). A possible effect of HHP processing on the characteristics of virulence of certain pathogens, and its modification, has been described (Bowman & Bittencourt, 2008). The exposure of a bacterium to a technological process as HHP is a cause of stress, which may provoke an SOS response (post-replication system that repairs DNA damage or errors allowing replication), triggering a horizontal genetic transfer of antibiotic resistance and virulence genes (Aertsen, Van Houdt, et al., 2004; Aertsen, Vanoirbeek, et al., 2004).

HHP-processed products experience a significant extension of shelf-life. Nonetheless, HHP-processed foods are usually non-sterile and therefore must be refrigerated to maintain their sensory characteristics and microbiological stability. In these circumstances, evolution of survivors or recontaminants (particularly psychrotrophic pathogens) over the shelf-life period should be studied. Cells with sublethal damage, under appropriate conditions (nutrient-rich substrates, appropriate temperature and storage time), can be resuscitated (Bozoglu et al., 2004) and psychrotrophs such as *L. monocytogenes* can constitute a risk (Ritz, Pilet, Jugiau, Rama, & Federighi, 2006). The microbiological analyses performed to HHP foods must consider the possible presence of sublethally injured microorganisms, whose resuscitation requires the use of methodologies and non- selective culture media, rich in nutrients and incubated at temperature and for sufficient time to permit the repair of damage (Kalchayanand et al., 1994; Patterson et al., 1995; Ritz et al., 2006; Ulmer, Ganzle, & Vogel, 2000).

5.2. Kinetics and modelling aspects

Modelling applied to HHP processing can be a useful tool in any risk assessment process because of its potential and applicability in prospective simulation, optimization, monitoring and validation (Smelt, Hellemons, Wouters, & van Gerwen, 2002). Predictive models of bacterial inactivation can use the existing databases regarding the effects of pressure, holding time, temperature, and intrinsic factors of food. The first stage is modelling inactivation kinetics, to develop primary models of microbial destruction. When the inactivation rate of the cell population is displayed in relation to time, first-order kinetics are sometimes observed, especially when the conditions (pressure, temperature) are extreme and processing times very short (Basak, Ramaswamy, & Piette, 2002). In such cases it is useful to model a parameter (D value, similar to that used in heat processing) that expresses the time needed to reduce the microbial population by 90%, or similarly, in a logarithmic unit. These kinetics (logarithmic-rate inactivation) is in theoretical accordance with the destruction of "one key molecule or structure", for example DNA degradation, which would cause cell death. However, the survival semi-logarithmic curves are often non-linear and shoulders and/or tails are typical (Garriga et al., 2002), also for spores (Ahn et al., 2007; Rajan, Pandrangi, Balasubramaniam, & Yousef, 2006). When curves appear with shoulders (latency periods followed by inactivation phases), they are explained due to cellular damage repairing, which occurs at the beginning of processing, but when the capacity of cellular repair is surpassed, counts decrease exponentially with the duration of processing. An additional explanation lays on the fact that microbial inactivation is due to an injury accumulated in the cell. When several key molecules should be inactivated to produce cell death, and a certain threshold is surpassed, it causes inactivation and cell death exponentially (Hoover et al., 1989). On many occasions, charts display tails which may be explained by the existence of sub-populations of cells particularly resistant to processing, due to better defence and repair systems against the stressor (Smelt, 1998). Other factors such as heterogeneous treatments, protective effects of dead spores, spore clumping and multiple inactivation mechanisms have been also pointed out (Cerf, 1977).

To fit these non-linear kinetics, many models have been used, including non-linear models such as biphasic, nth order kinetics, Gompertz-modified, Baranyi, or log-logistic models. The probabilistic models (Weibull) assume that the lethality is a result of likelihood and that destruction curves are a representation of the cumulative distribution of lethal events (Buzrul & Alpas, 2004; Buzrul, Alpas, & Bozoglu, 2005; Kingsley et al., 2007; lek Avsaroglu, Buzrul, Alpas, Akcelik, & Bozoglu, 2006; Panagou, Tassou, Manitsa, & Mallidis, 2007; Rajan, Ahn, et al., 2006; Van Boekel, 2002). From the Weibull model, the scale factor (b) and the shape factor (n) describe the extent of inactivation and degree of curvilinearity, respectively. For combined pressure-heat treatments, it has not yet been disclosed how pressure-thermal variables affect Weibull model parameters (Rajan, Pandrangi, et al., 2006). Current models do not usually take into account the come-up time as a variable in microbial inactivation, although significant spore reduction during the come-up time has been reported (Ahn et al., 2007).

Secondary models are obtained once a parameter that measures the baro-inactivation (D value, b value or similar) has been calculated, whereby the next step is to model its variation depending on intrinsic or extrinsic factors. There are plenty of models such as polynomial equations, square root model, artificial neural networks (Esnoz, Periago, Conesa, & Palop, 2006), or classic models such as Arrhenius or log-linear (Buzrul et al., 2005; Bull et al., 2005).

Evaluation of the impact of an HHP process on food microflora can be greatly helped by the development of adequate time-temperature-pressure integrators (TTPIs). These are pressure sensitive components which can be included in the food to be processed, and allow quantifying the impact of the process on product

safety (Fernandez Garcia et al., 2009; Mehauden et al., 2007; Van der Plancken, Grauwet, Oey, Van Loey, & Hendrickx, 2008).

5.3. Industrial applications

For microbiological risk assessments related to HHP food products a case-by-case evaluation has been advocated (Eisenbrand, 2005). Specific microbiological risks derived from high pressure-treated foods have not been identified, but the inactivation by HHP treatment of undesirable microorganisms present in a raw material has to be examined in each individual instance. For the development and evaluation of processes it is therefore necessary to characterize the hygiene-relevant key organisms specific to each food product (Eisenbrand, 2005). Evaluation of microbiological risks in an industrial context includes many other aspects such as the hazards present in the raw material, intrinsic factors of the foodstuff, packaging materials and extrinsic factors prior to processing, and during storage and distribution.

For those processes in which the HHP stage has a main technological implication and does not replace another preservation stage, the microbiological risk is low or very low. From a microbiological point of view, advantages will be obtained due to the reduction of microbial load of the product although this may not be the main objective. Examples are milk processing to make cheese (Trujillo et al., 2000), or seafood processing for meat extraction (Murchie et al., 2005). Likewise, the risk is minimal in those processes in which a stabilization of food is required to avoid the modification of the sensory characteristics during its storage, distribution and sale. This process can be useful in some fermented products such as beer, wine or milk products (Castellari, Arfelli, Riponi, Carpi, & Amati, 2000; Mok et al., 2006).

A widespread application in food processing is for RTE foods which are sliced after processing and packaging, to control the contamination introduced in this stage. Microorganisms to consider would be *L. monocytogenes* and other post-processing contaminants, whose prevalence would depend on the hygienic conditions of process, equipment and personnel. Risks can be minimized through a prior assessment of the conditions of HHP operation and adequate hygienic control of slicing and packaging. For fermented meat products such as sausages, if adequate starters are used (baroresistant, antimicrobial producers) they can remain in the product, affording additional protection. The previous packaging of products to be treated is a safety factor because it prevents recontamination.

A careful, comprehensive risk assessment should be performed for products with reduced levels of preservatives (such as nitrite, salt or other antimicrobials), minimally processed foods or products meant to be eaten raw or undercooked (e.g. bivalves). Shelf-life extension or food safety improvement can be achieved through combined process strategy. The use of combined treatments is an appropriate strategy to reduce the intensity of the treatments, thereby obtaining foods with characteristics close to fresh foods (less acidification, fewer preservatives), long shelf-lives and high levels of safety. The storage conditions, bearing in mind that these products have extended shelf-life, can allow the growth of baroresistant bacteria, and therefore the processing conditions should be carefully studied.

The risk assessment should be extremely careful if an HHP step is intended to reduce the intensity or replace a heat treatment, or when the product must achieve sterile conditions, which require the combination of heat processing with high pressure for the elimination of spores present. For low-acid, shelf-stable foods, *C. botulinum* is the most important microbiological hazard and the critical target in the calculation of appropriate process conditions. Before commercial application of the high pressure thermal processing, its effectiveness against pressure-resistant pathogenic spores should be thoroughly assessed.

6. Conclusions and future perspectives

As it has been shown, one of the main benefits obtained by using HHP in food processing is the extended shelf-life, accompanied by an increase in the safety of the product (Garriga, Grebol, Aymerich, Monfort, & Hugas, 2004; Hugas et al., 2002). The increase in shelf-life allows a significant extension of the best-before dates, whose extent depends on product characteristics and processing conditions. In packaged products with low microbial counts due to previous treatments (e.g. heating), HHP process can provide a high level of security. As mentioned, most vegetative pathogens are susceptible to pressure, and standard treatments achieve significant reductions. At international level agreement has been reached on standardized conditions for heat treatments (pasteurization, sterilization) according to certain time-temperature combinations. Likewise, the development of HHP would be favoured if standardized treatment conditions were proposed by international bodies such as *Codex Alimentarius*, ICMSF (International Commission on Microbiological Specifications for Foods) or ILSI (International Life Sciences Institute). The so-called Process Criteria (combinations of pressure-time-temperature for different foods and pathogens) can be calculated by predictive microbiology procedures, and would result from global values known as Food Safety Objectives (FSO), set for food–pathogen combinations. The establishment of these parameters would facilitate the development of procedures of Quantitative Microbiological Risk Assessment (QMRA) (Smelt et al., 2002). Food companies can incorporate the stage of HHP processing in HACCP plans, as a Critical Control Point, which can be monitored, optimized, validated, and for which critical limits and corrective actions may be set.

There are many areas in which research is being conducted in order to increase the effectiveness of HHP processing or to investigate the nature of the damage in the microbial cell (Balasubramaniam & Farkas, 2008; Torres & Velazquez, 2005). There is interest in using a combination of treatments such as antimicrobial substances included in the product and pressurizing gases such as carbon dioxide (Hong & Pyun, 1999; Park, Park, & Park, 2003; Watanabe et al., 2003). The optimization of stages prior to processing, the use of processing cycles, pressure pulsing, or combined heat–pressure processes to obtain shelf-stable products by enhancing the inactivation rate (especially for spores) are also important research issues. Other areas of interest are the development of modelling tools in predictive microbiology or QMRA, the use of (enzyme-based, physical) markers as process indicators, and the use of intelligent, active packaging or the impact of HHP on package materials. There has also been renewed interest in knowing the characteristics, physiology and taxonomy of so-called extremophile microorganisms, including the obligate barophile bacteria. Among the isolated species are Gram-positive bacteria such as *Carnobacterium*, and Gram-negative such as *Pseudomonas, Shewanella, Moritella, Colwellia* and *Photobacterium*. The strains possess characteristics as halophilia (Kaye & Baross, 2004), abundance of unsaturated fatty acids (Allen, Facciotti, & Bartlett, 1999; Yano et al., 1998), supercoiling or condensation on the DNA strands, with high efficiency catalytic enzymes and reduced enthalpy, and high amounts of helicase and cold-shock proteins. Knowledge of the physiological aspects of microorganisms will allow optimization and improvement of the effectiveness of HHP treatments. At the molecular level, deeper knowledge of the molecular basis of injuries, the behavior of bacterial membrane proteins under pressure and the possibility of changes in the pattern of virulence or other phenotypic properties would be very advantageous (Malone et al., 2006).

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