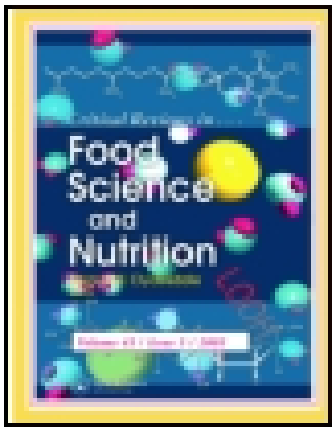

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Factors Affecting Bacterial Inactivation During High Hydrostatic Pressure Processing of Foods - a Review

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FACTORS AFFECTING BACTERIAL INACTIVATION DURING HIGH HYDROSTATIC PRESSURE PROCESSING OF FOODS – A REVIEW

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ABSTRACT

Although, the High Hydrostatic Pressure (HHP) technology has been gaining gradual popularity in food industry since last two decades, intensive research is needed to explore the missing information. Bacterial inactivation in food by using High Hydrostatic Pressure (HHP) applications can be enhanced by getting deeper insights of the process. Some of these aspects have been already studied in detail (like pressure, time and temperature, etc.), while some others still need to be investigated in more details (like pH, rates of compression and decompression etc.). Selection of process parameters is mainly dependent on type of matrix and target bacteria. This intensive review provides comprehensive information about the variety of aspects that can determine the bacterial inactivation potential of HHP process indicating the fields of future research on this subject including pH shifts of the pressure treated samples and critical limits of compression and decompression rates to accelerate the process efficacy.

Keywords: High Hydrostatic Pressure (HHP), bacterial inactivation, effect of matrix, compression rate, decompression rate, effect of microbial strains.

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INTRODUCTION

The application of High Hydrostatic Pressure (HHP) as emerging technology in food processing has steadily increased during past 20 years and has received particular attention as a viable alternative (economically and technologically) to thermal processes (Patterson, 2005). Several products, such as high-pressure-pasteurized orange juice (France), cooked ham (Spain), oysters (UK, USA), and fruits (Germany) have been evaluated with common agreement that the HHP 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).

Processing of foods by HHP offers unique advantages over traditional thermal treatments, as it exerts anti-microbial affects without changing the sensory and nutritional quality of food (Juan et al., 2007). One of the principal advantages of the HHP process is the expanded shelf-life and improvement of food safety due to large inactivation of microbial population. The other major advantages of HHP for processing and preservation of foods are elimination or significant reduction of heating, thus avoiding thermal degradation of food components; high retention of flavor, color and nutritional values; uniform and instant treatment of the product under pressure; reduced requirement for chemical additives; and potential for the design of new products due to the creation of new textures, taste and functional properties (Datta & Deeth, 1999).

In spite of remarkable advantages, HHP processing is still not widely accepted by food manufacturers. That is because of high initial cost, limited research information and lack of technical guidance to the manufacturers. On the other hand, important unsolved scientific and technological problems restrain wide scale application of HHP in food industry. With respect to

microbiological safety, quantifying inactivation of important food related pathogens by HHP is most urgent and critical in the establishment of high pressure processing. Although the number of kinetic studies on HHP inactivation of microorganisms is steadily increasing, a number of issues remain unresolved.

High pressure technology should be considered as an additional option for preserving and/or processing raw materials, intermediate constituents or complete foods, as there are already in the market a significant number of HPP-treated products thanks to the existence of industrial equipments from different manufacturers. Moreover, much research is being conducted by groups located in different research institutes and universities in many countries. These works covered a very wide range of aspects of applying high pressures: from the improvement and maintenance of equipments to microbiological, physicochemical, sensory and nutritional aspects of components and foods, as well as its economic aspects and environmental impact.

HHP is based on the application of two physical principles: Le Châtelierôs principle and the principle of pressure transmission in a uniform and instantaneous manner. According to Le Châtelierôs principle if a chemical system at equilibrium experiences a change in concentration, temperature, volume, or partial pressure, then the equilibrium shifts to counteract the imposed change and a new equilibrium is established. In addition to these principles, there are some factors that may affect the bacterial inactivation by HHP. Processing of food products by high pressure obviously implies a much higher pressure than the atmospheric one, usually in the range of 500ï1000 times higher. It will, therefore, be essential to note that changes in the components will follow the thermodynamic constraints that involve working at a given pressure, which implies that one of the basics to keep in mind is that componentsô phase-changes will not follow

the conditions (thermal, volume) which are usually considered when working at 0.1 MPa. Therefore, an extrapolation from what happens in many processes at atmospheric pressure to what is happening in high pressure should not be expected. Changes that imply a positive variation in volume will be hindered when increasing pressure, and in the same way elevated pressure will favor processes that involve changes associated with a reduction in volume, which is known as Le Châtelierôs principle. As water-phase changes (dissociation constant) are clearly different under pressure, chemical balances shift to different equilibrium values depending on pressure value (Mor-Mur and Saldo, 2012).

In present work, research information has been reviewed about effect of different factors that should be considered during application of HHP in food processing. Some of these aspects are still under investigation to discover the unknown facts and also to enhance the microbial inactivation efficiency of HHP treatments.

1- PRESSURE, TEMPERATURE & TIME MODELS

1.1-Pressure Level and Holding Time

Research has revealed that bacterial inactivation is directly proportional to the pressure level and pressure holding time. Earnshaw (1995) studied the inactivation of *Staphylococcus carnosus* by HHP in nutrient broth. He found the reductions of 0, 0.5, 1.5 and 2.5 log units at 300, 400, 500 and 600 MPa respectively, at 20 ºC for 30 minutes. Alpas et al. (2000) reported that increasing pressurization time from 5 to 10 min at 207 MPa, had significant effect on inactivation of *S. aureus* and *L. monocytogenes* suspended in Tryptone Soy Broth with yeast extract.

Yuste et al. (2004) while working with *S. aureus* found that among different combinations of pressure and time during HHP treatments, in some cases, time influences the level of inactivation more than pressure.

Chen et al. (2012) studied the microbial survival parameters under HHP. They developed optimization algorithm to estimate survival parameters through finding the best fitting-curves to non-isobaric survival data. They used published data on *E. coli* ATCC 25922 to test the algorithm, and concluded that the optimization algorithm developed provides an accurate estimation of microbial survival parameters from non-isobaric survival data, presented in terms of microbial log reduction versus time and corresponding pressure profiles used to achieve that inactivation. Although the alogrithm was tested by using Weibull model, it should be applicable to other inactivation kinetic models since a specific inactivation kinetics model does not need to be assumed.

A number of authors proposed first-order inactivation kinetics for various bacteria (Mussa et al., 1999; Ponce et al., 1998). Gervilla et al., (1999) treated *Staphylococcus aureus* in ovine milk at different pressure-time combinations. They reported a linear decline in *S. aureus* log-count with increase in pressure level and holding time. Pressure studies (Erkmen & Dogan, 2004; Erkmen & Karatas, 1997; Van Opstal et al., 2005) indicated that at a given pressure, counts of microorganisms, including *S. aureus* (Figure 1) and *E. coli* O157:H7, decrease exponentially with holding time, and a decimal reduction time (*D* value) can be calculated for each organism. For example, pressure inactivation of *E. coli* MG1655 was investigated at 256 different combinations of pressure ranging from 150 to 600 MPa and temperatures in the range of 5 ï 49.5 ºC. In fresh carrot juice, linear relationship was suitable to fit the inactivation, in $\log_{10}$ values,

and treatment times under all pressure-temperature combinations (R^2 ï values > 0.91) (Van Opstal et al., 2005).

On the other hand, also significant deviations from linearity have been reported, sometimes for the same bacteria and food matrix (Buzrul, et al., 2008; Chen & Hoover, 2003; Tassou et al., 2008). The inactivation curves induced by milder pressure treatment rarely obey first-order model, and tailing is frequently observed. (Patterson et al., 1995). Typically, isobaric survival curves exhibit a tail (Ahn et al., 2007; Margosch et al, 2006; Panagou et al., 2007; Rajan et al., 2006 ; Tossou et al., 2008 ; Van Boejen et al., 2008). It can be described by either Weibull model or other nonlinear models (Ahn et al., 2007; Celik et al., 2009; Chen, 2007; Tassou et al., 2008; Van Boejen et al., 2008).

Successful use of *D*-value for defining conditions of food pasteurization relies on the inactivation rate being log linear. Neverthless, *D*-value is often calculated from a linear portion in a non-linear survival curves (Pfeifer and Kessel, 1994). Noma et al., (2006) suggested that the presence of genetically pressure-resistant subpopulation was responsible for tailing. Accorging to the authors, cytoplasmic membrane of the tail-culture cells had higher stability to pressure treatment at 100 MPa for 10 min than that of the original cells. It has been further discussed under physiological state of microorganisms.

An effective pressure inactivation of microorganisms under mild conditions requires the elucidation of the mechanism of tailing and the potential means to avoid tailing.

1.2-Treatment Temperature

Temperature is another important factor that affects the inactivation level of vegetative bacteria. It is well know that the temperature of the product and pressure fluid can affect microbial resistance, with larger inactivation rates obtained above or below the ambient temperature. In fact, elevated temperature (30-50 ºC) promotes pressure inactivation of microorganisms but the effect of low temperature (<20 ºC) on inactivation is still not clear (Alpas el at., 2000). Therefore the process efficacy is influenced by the processing temperature (Alpas et al., 2000; Bayindirli et al., 2006). Bacterial cells are relatively less sensitive to hydrostatic pressure at 20 - 35 ºC, but become more sensitive to pressurization above 35 ºC, due to phase transition of membrane lipids (Kalchayanand et al., 1998). Cheftel (1995) correlates the decrease in resistance to pressure at low temperatures with changes in membrane structure and fluidity through weakening of hydrophobic interactions and crystallization of phospholipids. Moderate heating (40 ï 60 ºC) can enhance microbial inactivation by pressure (Carlez et al., 1993), which in some cases makes it possible to get desired results at lower pressures. Patterson and Kilpatrick (1998) applied HHP to *E. coli* O157:H7 and *S. aureus* in milk and poultry. Their findings showed a practical necessity for combined use of pressure (400-500 MPa and elevated temperatures (50 ºC) for 5-6 $\log_{10}$ (cfu/g) reduction.

Pressure-temperature combinations that lead to a 5 log reduction of several pathogenic and spoilage organisms within 5 min of treatment are exemplarily shown in Figure 2. It is generally accepted that pressure and temperature act synergistically on the destruction of vegetative bacteria in the high temperature domain, which is indicated by the left end of isorate curves in Fig. 2 (Buckow and Heinz, 2008).

High process temperatures are mainly required when spore inactivation is concerned. The term Pressure Assisted Thermal Sterilization (PATS) is generally used while dealing with inactivation of bacterial spores (Wimalarante and Farid, 2008). Unlike vegetative cells, spores can not be killed at low temperatures during HHP processing. Bacterial endospores, as compared to vegetative cells, display a considerably higher resistance to temperature and pressure. Spores of *Clostridium botulinum* and *Bacillus* species are the key bacteria for the safety or the spoilage of low acid (heat treated) preserved goods. These spores have shown remarkable tolerance to pressures above 1000 MPa at room temperature (Margosch et al., 2006; Margosch et al., 2004). On the other hand, many other bacterial endospores, which are relevant to food, are inactivated at pressures of 600 MPa or higher in combination with an initial temperature above 60 °C (Heinz & Knorr, 2002). Often the required inactivation temperature and/or time is lowered by combination with pressure as indicated in the pressure-temperature plain of Fig. 3 for a number of bacterial spores (Buckow and Heinz, 2008).

In dependence of the applied temperature and pressure level, bacterial endospores pass through different physiological pathways, depending on the temperature and pressure level, and this can induce spore germination or a subsequent inactivation during the treatment. In literature two- (Margosch, et al., 2006) or three-step models (Mathys, et al., 2007) for spore inactivation are discussed, but it is assumed that the spore germination is the first step of spore inactivation under pressure (Gould & Sale, 1970).

Application of HHP results in pressure-induced germination of bacterial spores (Rendueles et al., 2011) and these germinated spores could be destroyed by simultaneous use of mild heating (Clery et al., 2004; Margosch et al., 2006). *Bacillus subtilis* spores were quite resistant to 600

MPa at 20 ºC but at 60 ºC and 70 ºC the inactivation of 0.5 and 3 log cfu/ml, respectively, was observed (Syed et al. 2012).

In addition, adiabatic heating is also an important aspect while discussing microbial inactivation by pressure. Different compression media exhibit varying characteristics of heating up during compression. Pressure transmitting fluids are used for uniform transfer of pressure to the food. Many of early laboratory machines were not made from stainless steel and necessitated the use of oils as the pressure medium. Solutions of castor oil, silicone oil, water and glycol are commonly used as pressure transmitting fluids. In general it is accepted that when water is used as compression medium, physical compression results in a temperature increase of 2-3 ºC per 100 MPa (Cheftel, 1995). When organic solvents or oils are employed as pressure transmitting fluid, the temperature increase is greater than that of water due to their higher compressibility, lower thermal conductivity and lower heat capacity (Makita, 1992).

Difference between compressibility of compression fluid and the food sample may result in heat transfer between compression fluid and food sample and also from compression fluid to vessel walls. The potential influence of adiabatic heating characteristics of compression media on microbial inactivation is not investigated in detail. Balasubramanian & Balasubramaniam (2003) studied the adiabatic heating characteristics of different concentration of water-glycol mix. They reported that pressure transmitting fluid containing highest percentage of glycol (25:75 water-glycol) showed highest temperature increase.

The apparent temperature increase in compression medium during HHP is influenced by the target pressure, holding time, product compressibility, initial temperature and rate of heat loss to surroundings. Change in compression fluid temperature as a result of adiabatic heating and

subsequent heat transfer may point out the risk of deviation from isothermic conditions and consequently the risk of non-uniform treatment, therefore should be considered carefully during HHP microbial inactivation studies. Adiabatic heat transfer to food samples and equipment vessels depends upon the sample size, packaging material, thickness and type of packaging material, thickness of equipment vessel and its heat conduction characteristics.

2-RATES OF COMPRESSION AND DECOMPRESSION

There has been very limited work done on this aspect of HHP and only a few references are available regarding the effect of rate of pressurization and depressurization on inactivation of bacterial cells. The available literature on this topic is quite contradictory and there is room for more research on this aspect.

The issue was highlighted by Smelt (1998) who assumed that a slow ramp during compression might induce a stress response of microbial cells and hence leads to a lower inactivation effect of the process. In the same way, Herdegen (1998) reported that a process of rapid pressurization (6.7 MPa/s) and slow decompression (0.83 MPa/s) was more effective than a process of same maximum pressure and holding time but reverse pressurization and depressurization rates, as cited by Rademacher et al. (2002).

Noma et al. (2002), investigated the inactivation and injury effects of HHP treatments at different pressure levels (from 70 to 400 MPa) combined with slow and fast decompression (30 s and 1 ms, respectively). The authors concluded that a very rapid decompression procedure could enhance the injury, which causes the higher bactericidal effect of HHP treatments. On the other hand, Rademacher et al. (2002) investigated effect of pressurization ramp on inactivation kinetics

of *Listeria innocua* suspended in Tris buffer by using fast pressurization ramp (8.3 MPa/s) and slow depressurization ramp (1.7 MPa/s) in comparison with slow pressurization (1.7 MPa/s) and fast depressurization (8.3 MPa/s). They concluded that rate of pressurization and depressurization in the range of 1.7-8.3 MPa/s, does not affect inactivation kinetics of *L. innocua*, if the temperature changes are negligible during pressure treatment.

While working with inactivation of *Bacillus* spores Syed et al. (2012) found that processing of 600 MPa at 60 & 70 ºC was more lethal when slow compression and slow decompression rates were used. The increased lethality of slow rates was more likely linked to increased treatment times. However, combination of fast compression with slow decompression resulted in highest number of injured/germinated population.

Some controversial results on the effect of pressure increase rate can be linked to the dissipation of the adiabatic heat generated. In non-accurately designed experiments the effect of fast pressurization may be hindered by the effect of accumulation of heat generated adiabatically, while in slow compression the heat generated may be released and does not cause a high temperature increase as in the case of fast compression. Therefore, equipment performance and operation procedures may lead to differences in the log reductions of the same strain in a similar food (Mor-Mur and Saldo, 2012).

Rupture of bacterial cells due to pressurization and depressurization is considered to be the main cause of destruction of microorganisms under different pressure regimes (Yano et al., 1998). Ramaswamy et al. (2008) discussed the effect of pressurization and depressurization as pulsing effect. This pulse effect was explained to be due to pressurization (adiabatic compression) and depressurization (rapid expansion) that leads to rupture of cells and microbial death.

Although these results give an idea that there is difference in bacterial inactivation by changing rates of compression and decompression, but the issues still unclear are the critical limits of compression and decompression rates. It has been reported that changing rates of compression do not have linear effect on bacterial inactivation (Syed et al. 2013; Rademacher et al. 2002); rather there would be some threshold point beyond which the inactivation efficiency of process is enhanced significantly. Those threshold limits need to be investigated for each microorganism. Furthermore, development of mathematical models to estimate the resultant inactivation of bacteria by changing rates of compression and decompression also needs to be worked out.

3-MICROBIOTA

It is generally accepted that efficiency of any treatment for bacterial inactivation is directly related to initial microbial population in the subject matrix. The high initial microbial load always results in high survival counts after treatment. Hence, initial microbial count in any matrix is a primary factor that can reduce the effectiveness of any treatment. Mostly, to overcome this issue some pretreatments like initial staining and hygienic measures are practiced.

HHP 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, et al., 2001). In addition, microorganisms, including pathogens, can vary significantly in their responses to high pressure and this variation exists not only between different species but also between strains of the same species. Certain strains of *E. coli* O157:H7 were found to be particularly resistant to pressure, in a variety of substrates (Benito et al., 1999). It is demonstrated that the pressure resistance of certain natural isolates of *E. coli* O157:H7 varied

greatly (Robey et al., 2001). Among the pathogenic non-spore forming gram-positive bacteria, *L. monocytogenes* and *S. aureus* are the two well studied pathogens regarding the use of HHP processing. *S. aureus* appears to have high resistance to pressure (U.S. FDA, 2000).

3.1- Gram-positive Vs Gram-negative Bacteria

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 et al., 1991) but there is considerable overlap. For instance, *L. dextranicum* is more sensitive to HHP than several gram-negative bacteria (*S. flexneri, E. coli* and *S. typhimurium*), and the pressure resistant mutant of *E. coli* (LMM1010), is more resistant than several gram-positive bacteria (*L. dextranicum, L. innocua*, and *L. plantarum*), particularly at pressures >300 MPa (Wuytack et al., 2002).

3.2-Physiological State of Microorganisms

Experiments in model systems show that the physiological status of microbial populations subjected to HHP processing influence pressure resistance. Vegetative cells in the growth phase are normally more sensitive to HHP than are cells in stationary phase (Manas & Mackey, 2004; Hayman et al., 2007). Chen (2007) worked with the HHP treatments of six food-borne pathogens suspended in UHT whole milk. The results indicated that small subpopulations of bacteria were much more resistant than the rest of the populations. The author explained that there may be heterogeneous sensitivities to pressure within the bacterial populations caused by different

physiological states within the population. Similarly, cocci are more resistant than rods (Yuste et al. 2004).

The presence of heat and cold-shock membrane proteins 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 affects the degree of resistance to pressure (Malone et al., 2006; Wemekamp-Kamphuis et al., 2004).

In food industries, the foods do not always contain a previously known population of pathogenic bacteria and their physiological state. So any standard treatment of HHP may leave a risk of viable pathogens and lead to serious consumer hazards. This may be one of the reasons for limited adaptation of HHP in food industries especially in liquid foods. The development of HHP treatment to produce commercially sterile product for all food categories might be a challenge for upcoming research groups.

4-TYPE OF MATRIX

Studies on inactivation of bacteria by HHP treatment usually showed that the inactivation of bacteria in food system was more difficult than in water or buffer system (Patterson et al. 1995).

4.1- Food Composition

The chemical composition of food is important, since the presence of fats, proteins, minerals and sugars serves as a protector and increases microbial resistance to HHP (Black, Huppertz, et al., 2007; Molina et al., 2004). Several authors reported that bacteria are more resilient in complex matrix as milk or meat, compared to a buffer at the same pH. Chen and Hoover (2003) observed

a strong baroprotective effect on *Yersinia enterocolitica* in whole milk than in phosphate buffer when treated at 350-450 MPa at 22 ºC for 10 min. Microorganisms generally show higher resistance in food systems and some organic matters contribute to that resistance.

In addition, as to the effect of fat on inactivation of microorganisms, contradictory information is found in the literature. Some authors found a baroprotective effect of fat on inactivation of vegetative bacteria that increased with fat content (Styles et al., 1991; Garcia-Graells et al., 1999), while others did not observe this difference (Garcia-Risco et al., 1998; Gervilla et al., 2000). Gervilla et al. (2000) inoculated different microorganisms (*E. coli, Pseudomonas fluorescens, L. innocua, S. aureus* and *Lactobacillus helveticus*) in Ringer solution and milk samples with 0, 6 and 50% fat contents and treated with 100 to 500 MPa pressure treatments at 4, 25 and 50 ºC. They reported that ovine milk with 0, 6 and 50% fat had baroprotective effect as compared to Ringer solution, but the samples with 6 and 50% fat did not show a progressive baroprotective in all pressurization conditions or for all microorganisms. Escriu and Mor-Mur (2009) did not found any correlation between the quantity and even the quality of fat (fat saturation level) and the destruction of *Listeria* and *Salmonella* in meat model systems. Both of the microorganisms exhibited mixed tendencies with different fat quantities and qualities.

Similar studies for varying levels of sugars, proteins and other food constituents need to be carried out for deeper insights of the HHP treatments. Existing studies only provides information about the protective effect of single levels of food constituents against HHP, but the comparative studies with varying levels of different food constituents are missing. Such studies will help to categorize foods for high and low pressure requirements.

4.2- pH / Acidity

The pH is another major stress factor that can bring variability in the resistance for different microorganisms (Stewart et al., 1997). Although pH alone may not be enough to inactivate microorganisms, its combination with high pressure greatly increases treatment lethality. After pressure processing, the injured cell cannot heal, and even those not showing any effect of the treatment may become more sensitive to the high acidity of medium. The dependence of the effectiveness of the pressure treatment on pH has been studied by many researchers, showing that more acidic conditions tend to enhance the effect of pressure. This linkage between high pressure and low pH is one of the examples of hurdle theory. This dependence has been evidenced in bacteria (Ritz et al., 2006) and even in viruses (Kingsley and Chen, 2009).

As pH changes under pressure are difficult to be measured, very little is known on the real effect of pH during HPP. As different buffering systems show different pH-shifts under pressure, Mathys et al. (2008) conducted experiment using different buffering systems, showing a higher inactivation of *Geobacillus stearothermophylus* spores by pressure when suspended in phosphate buffer than when suspended in ACES buffer (pH 4.5 ï 8), due to the fact that phosphate has especially high dependence on pressure, thus lowering pH during treatment.

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, leading to inability to maintain the homeostasis (Hoover et al., 1989). A previous adaptation to environmental conditions can modify susceptibility, and cells of *L. 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).

The pH variations under pressure have been scarcely studied, although the interaction of pH-pressure is well known. There is a shift in the dissociation equilibrium with a different intensity depending on the buffering system. On dairy systems the pressure produces a permanent change on pH caused by a shift in calcium equilibrium. For transient changes the information of the process is difficult to be measured, as the instrumentation suitable to withstand high pressure is limited. Several studies had assayed dyes to measure pH changes through optical methods (Hayert et al., 1999, Salerno et al., 2007); however, the problem to measure pH under pressure in actual foods still remains unsolved (Mor-Mur and Saldo, 2012).

Keeping view the quite evident role of pH in bacterial inactivation and its susceptibility to change under pressure, there is need to develop HHP equipments with pH probe that can be immersed in to the sample and measure continuous pH variations of the food sample under pressure. Such type of development may provide process insights for better control on HHP treatments.

4.3-Water Activity (a_w)

Water content plays a crucial role in determining cellular damage under pressure with a strong relationship with water activity: the lower the a_w of the food matrix, the higher the protection of spoiling and pathogenic agents against HHP effects, so lower values of water activity (a_w) increase microbial resistance to HHP (Black, Huppertz et al., 2007; Black, Setlow, et al., 2007; Hayman et al., 2008; Patterson, 2005). For *L. monocytogenes*, Hayman et al. (2008) postulated that low a_w results in protein stabilization, which prevents protein denaturation and cell death during HHP. Moussa et al. (2009) studied the effect of a_w between 0.11 and 0.99 in *S. cerevisiae*

and found that a_w Ò0.71 completely prevented the cell inactivation, as when water is available in a sufficient amount high pressure could induce membrane permeability causing uncontrolled mass transfers that could lead to death. 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 (Raso et al., 1998). This fact also applies to spores such as *B. cereus* and can be attributed 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) due to effect on osmotic pressure.

5- DESTRUCTION VS SUB-LETHAL INJURIES

After HHP treatments one population of microorganisms may be killed, another population may survive, and a third population may be sublethally injured (Wu et al. 2001). In addition, a small population of bacteria has been reported to be stressed, that are not able to exhibit growth immediately after HHP treatments but during subsequent storage they can grow normally. Stress is a change in the environment that imposes either reduced growth or survival potential. For any stress, the bacterial cell has a defined range within which the rate of colony forming units (CFUs) is positive (growth), zero (survival) or negative (death). In microbial populations, healthy cells are usually countable on both nonselective and selective media, whereas stressed cells are able to form colonies on nonselective media but not countable on selective media (Comas and Rius, 2009). Injured microorganisms can be distinguished from stressed ones by their inability to grow in specific media after a certain period of stress release. A series of

morphological and structural changes in the cell, such as the separation of membrane from cell wall, the lengthening of the cell, the compression of gas vacuoles (Patterson, 2005) and the condensation of nuclear material (Manas and Mackey, 2004; Wouters et al., 1998) are described. Injured and stressed organisms are potentially as important as their normal counterparts because they can resuscitate and become functionally normal in a favorable environment with consequent danger to public health. During repair, restoration of growth capabilities will occur before normal growth occurs. Many cellular modifications are reversed and losses of cell constituents are restored to the normal state during incubation.

Syed et al. (2013) worked with *E. coli* in Tris buffer, skimmed milk and orange juice at 600 MPa/ 3 m). They found higher recovery of stressed *E. coli* cells after 24 h in Tris buffer than in milk (1.19 and 0.79 log cfu/ml, respectively) during storage at 4 ºC. However, samples of orange juice (non favorable environment because of low pH) did not allow stressed cells to recover.

The availability of some substrates or the presence of factors in the matrix such as vitamins and amino acids in the food (or growth medium) allows better recovery of sub-lethally damaged cells after HHP processing (Tassou et al., 2007). It has also been described that the kind of solute (salt or sugar) can have significant influence on cell survival after processing and especially on the resistance of the spores (Patterson 2005). Cells with sub-lethal damage, under appropriate conditions (nutrient rich substrates, appropriate temperature and storage time), can resuscitated (Bozoglu et al., 2004) and psychrotrophs such as *L. monocytogenes* can constitute a risk (Ritz et al., 2006). The microbiological analyses performed to HHP processed foods must consider the possible presence of sub-lethally 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 et al., 2000).

Mussa and Ramaswamy (1997) treated milk (inoculated with *L. monocytogenes*) at 350 MPa and observed an increase of 4 log cfu/ml after 12, 18 and 25 days of storage at 10, 5 and 0 ºC, respectively. They recommended refrigeration storage after HHP treatment to inhibit the rapid recovery of microorganisms.

Generally, most stressed cells repair within 2-4 h at a suitable incubation temperature in nutritionally rich non-selective medium. Moreover, the re-synthesis of RNA lost during injury is critical in the first stage of repair (Ray, 1986).

Research has indicated that regardless of the nature of the stress imposed on a microbe, for injured vegetative cells: (a) the injuries are repaired when incubated in an appropriate environment, (b) the optimum temperature and time differ with the nature of the stressor, (c) the completely repaired cells regain normal resistance to the selective agents in the media, and (d) the repair process precedes cell multiplication (Wu, 2008). Therefore, it is desirable to allow injured cells to repair any damage before enumeration by customary procedures (Ray and Adams, 1984).

CONCLUSIONS

In this review many factors have been discussed. Most of the HHP process parameters have already been discussed in detail, but some subjects are still incomplete or provide contradictory information. Bacterial inactivation efficiency of HHP is directly proportional to pressure level, holding time, treatment temperature. Selection of these parameters is interlinked with

physiological state of target bacteria i.e. vegetative or spore forms etc. The rates of compression and decompression effect the bacterial inactivation but need to be further studied to define the critical limits. The types and growth phases of microorganisms and type of matrix determine the effectiveness of HHP process. Changes in pH of under-pressure matrix are still unknown and need to be investigated. It is recommended that for efficient HHP processing food manufacturers should be well aware of their target microorganisms as well as chemical constituents of the foods and HHP processing parameters about pressure level, holding time, temperature as well as rate of compression and decompression should be selected accordingly.

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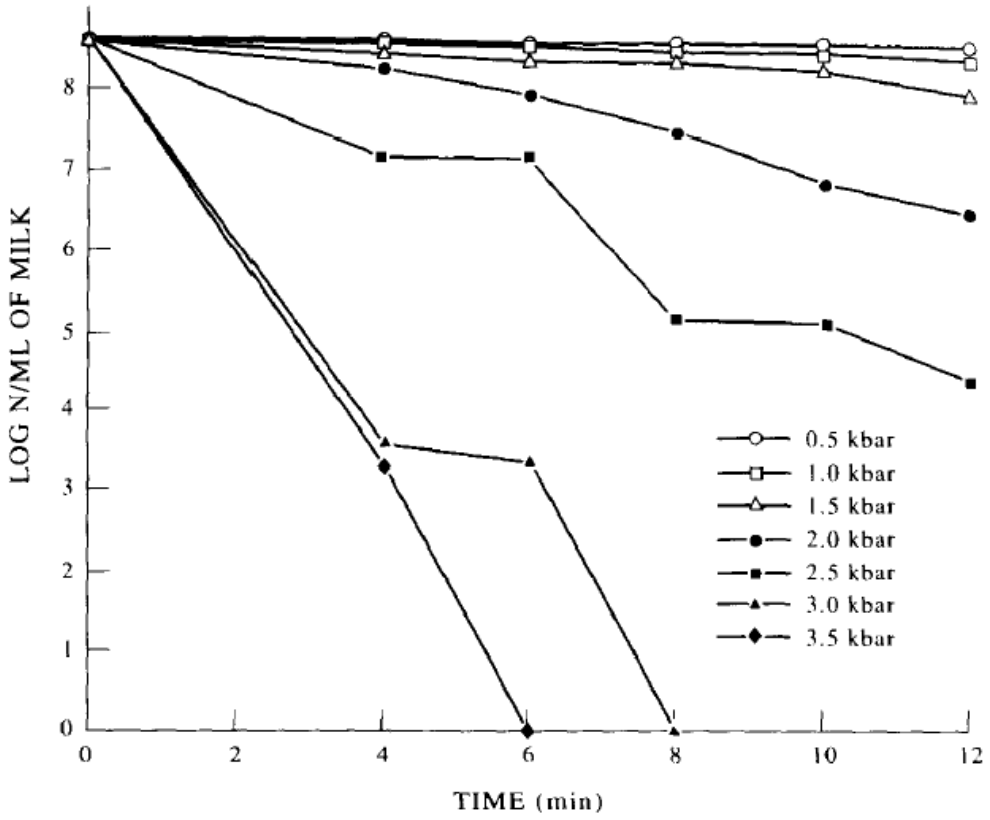

Figure 1. Inactivation of *S. aureus* in cowôs milk at different pressure vs time combinations at 20 ºC.

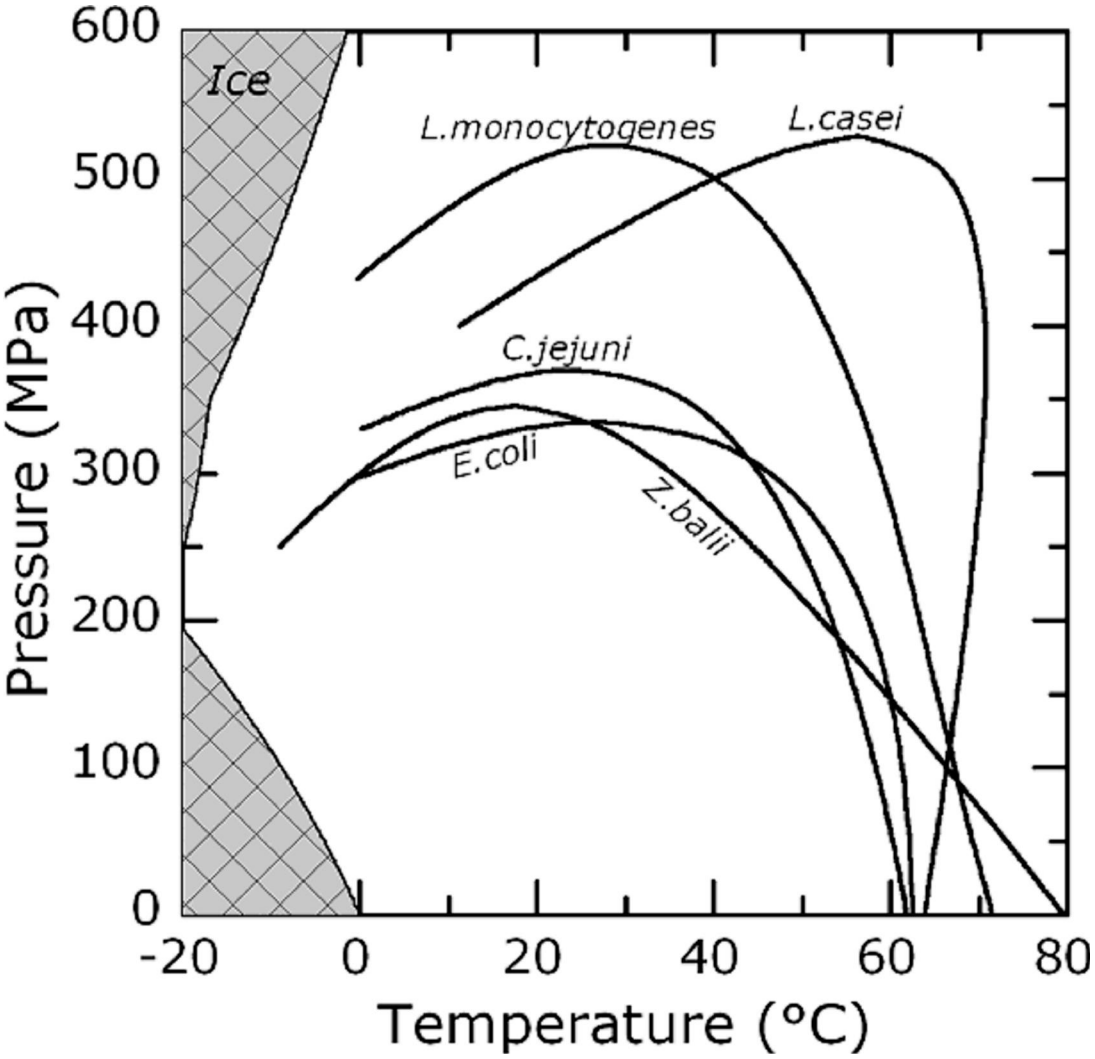

Figure 2. Pressure-temperature isorate diagram for 5 log inactivation of different vegetative bacteria after 5 min isothermal/isobaric treatment.

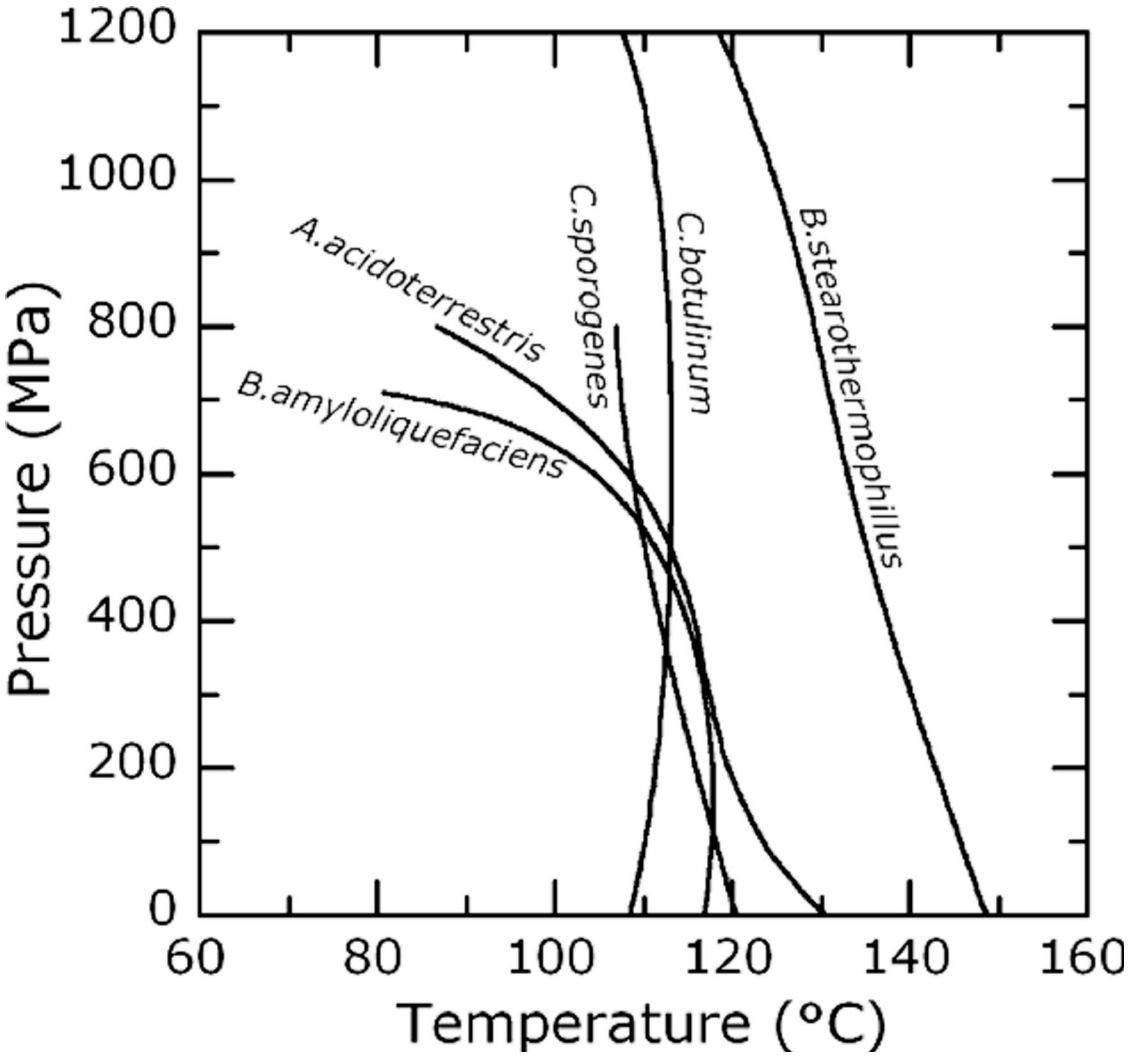

Figure 3. Pressure-temperature isorate diagram for 5 log inactivation of different endospores after 5 min isothermal/isobaric treatment.

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