Universidade de Aveiro Departamento de Biologia

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Efeito da taxa de pressurização na inativação de *Listeria innocua*

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Effect of pressurization rate on the inactivation of Listeria innocua

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, ramo de Microbiologia Clínica e Ambiental, realizada sob a orientação científica da Doutora Maria Ângela Sousa Dias Alves Cunha, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro, e coorientação do Doutor Jorge Manuel Alexandre Saraiva, Investigador Auxiliar do Departamento de Química da Universidade de Aveiro.

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palavras-chave

Processamento por alta pressão, taxas de pressurização e despressurização, *Listeria innocua*, segurança alimentar.

resumo

O processamento por altas pressões possibilita a inativação de microrganismos a temperaturas sub-letais garantindo a preservação das propriedades organoléticas dos alimentos. No entanto, é necessário que sejam determinadas e estabelecidas as condições ótimas para uma inativação eficiente, nomeadamente no que respeita ao valor de pressão a ser aplicado, tempo de pressurização e temperatura. A taxa de pressurização pode ser também uma condicionante da eficiência de inativação.

O presente trabalho teve como principal objetivo avaliar o efeito de diferentes taxas de pressurização na inativação de *Listeria innocua*. Para isso, culturas em fase estacionária em *Tryptic Soy Broth* (TSB) foram sujeitas a diferentes taxas de pressurização até uma pressão de 300 MPa ou 400 MPa, aplicada durante 1 e 5 minutos, à temperatura ambiente. Adicionalmente foi também avaliada a recuperação das culturas microbianas após tratamentos com alta pressão (400 MPa e 500 MPa), quando armazenadas a temperatura de refrigeração (4 °C) e temperatura ambiente (25 °C) durante 1, 5 e 10 dias. A concentração de sobreviventes foi avaliada por contagem de colónias após sementeira por incorporação em meio *Tryptic Soy Agar* (TSA) das diluições convenientes. O fator de inativação foi calculado como Log_{10} (N_0/N_i).

Os resultados mostram que existem diferenças no fator de inativação obtido com diferentes taxas de pressurização/despressurização, para tratamentos a 300 MPa durante 5 minutos. A inativação revelou-se significativamente mais eficiente com as taxas de pressurização e despressurização baixas (1,5 MPa s^{-1} e 3,2 MPa s^{-1}, respetivamente). Não houveram diferenças significativas no fator de inativação obtido nas restantes condições testadas – 300 MPa durante 1 minuto e, 400 MPa durante 1 e 5 minutos.

Os resultados referentes à recuperação da viabilidade das células após tratamento sub-letal a 400 MPa e tratamento letal a 500 MPa mostram recuperação completa da concentração inicial de células viáveis quando a cultura é armazenada a 25 °C. Nas culturas mantidas a 4 °C, após tratamento sub-letal o teor de células viáveis permanece estável, não havendo indícios de recuperação nem inativação tardia, pós-tratamento.

Os resultados permitem concluir que para além do valor de pressão e do tempo de pressurização, a taxa de pressurização é um parâmetro relevante na eficiência de inativação de *Listeria innocua* e que o fator de inativação permanece estável até 10 dias de conservação a 4 °C.

keywords

High pressure processing (HPP), pressurization and depressurization rates, *Listeria innocua*, food safety.

abstract

High pressure processing (HPP) enables the inactivation of microorganisms to sublethal temperatures ensuring the preservation of the organoleptic properties of food. However, must be determined and established the optimum conditions for an efficient inactivation, namely as regards the pressure value to be applied, holding time, and temperature. The pressurization rate can also be a condition of efficiency of inactivation.

The present study had as main objective to evaluate the effect of different rates of pressurization in the inactivation of *Listeria innocua*. For that, cultures in stationary phase of growth in Tryptic Soy Broth (TSB) were subject to different pressurization rates up to a pressure of 300 MPa or 400 MPa, applied during 1 and 5 minutes, at room temperature. Additionally it was also evaluated the recovery of microbial cultures after treatments with high pressure (400 MPa and 500 MPa), when stored at chill temperature (4 °C) and room temperature (25 °C) for 1, 5 and 10 days. The concentration of survivors was evaluated by counting colonies after pour-plated in Tryptic Soy Agar (TSA) of convenient dilutions. The inactivation factor was calculated as Log_{10} (N_0/N_i).

The results show that there are differences in the inactivation factor obtained with different pressurization/depressurization rates, to treatments at 300 MPa during 5 minutes. The inactivation has proven to be significantly more efficient with the low pressurization and depressurization rates (1.5 MPa s^{-1} and 3.2 MPa s^{-1}, respectively). There were no significant differences in the inactivation factor achieved in other conditions tested – 300 MPa during 1 minute, and 400 MPa during 1 and 5 minutes.

The results concerning the recovery of cell viability after sublethal treatment at 400 MPa and lethal treatment at 500 MPa show full recovery of initial concentration of viable cells when the culture is stored at 25 °C. In cultures maintained at 4 °C, after sublethal treatment, the content of viable cells remains stable, with no evidence of recovery neither late inactivation, post-treatment.

The results allow concluding that beyond the pressure value and holding time, the pressurization rate is a relevant parameter in the efficiency of inactivation of *Listeria innocua* and that the inactivation factor remains stable until 10 days of storage at 4 °C.

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Acronyms and Abbreviations

α	Significance level
ANOVA	Analysis of variance
ATP	Adenosine Triphosphate
a_w	Water activity
CCP	Critical control point
CFU	Colony forming-units
CFU mL^{-1}	Colony forming-units per milliliter
CO_2	Carbon dioxide
E. coli	*Escherichia coli*
FAD	Food and Drug Administration
FAO	Food and Agriculture Organization
GC content	Guanine and cytosine content
HACCP	Hazard Analysis Critical Control Point
HP	High pressure
HPP	High pressure processing
HTST	High-temperature short-time
L.	*Listeria*
LAB	Lactic acid bacteria
NaCl	Sodium chloride
NASA	National Aeronautics and Space Administration
NCTC	National Collection of Type Cultures
OD	Optical density
PEF	Pulsed electric fields
S. aureus	*Staphylococcus aureus*
S. enteritidis	*Salmonella enteritidis*
S. typhimurium	*Salmonella typhimurium*
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UHT	Ultra-high temperature
USA	United States of America
UV	Ultraviolet
UK	United Kingdom
WHO	World Health Organization

1. Introduction

1.1 Food safety

Over time and starting from the moment of harvest, foods lose their quality by physical, chemical, microbiological, or enzymatic reactions. The main agents involved in food deterioration are microorganisms and enzymes, being these key targets of preservation techniques. Such techniques are used to prevent food spoilage and increasing shelf life, as well as safety (Raso & Barbosa-Cánovas, 2003).

In recent years, many technological advances have been achieved by the food industry in relation to the control of pathogens of great importance to the sector. However, despite of a high level of innovation in preservation techniques, the number of incidents related to diseases transmitted by eating contaminated foods did not have an overall decrease, and food-borne diseases are still regarded as one of the greatest public health problems currently (Panisello & Quantick, 2001, Jofré, *et al.*, 2009). For decades, the main agents associated with food-related diseases were *Salmonella spp., Staphylococcus aureus, Bacilus cereus* and *Clostridium botulinum.* However, given the requirements of consumers for fresh products in recent decades, other microorganisms emerged, namely *Yersinia enterocolitica*, *Campylobacter spp., Listeria monocytogenes* and *Escherichia coli* O157 (Jofré, *et al.*, 2009).

The high incidence of food-borne diseases combined with i) greater knowledge and public awareness on the serious effects of food pathogens in human health and, ii) an increase of production and industrialization which, consequently, takes an increased risk of contamination of foods and increases the number of people exposed to the outbreak of such diseases, leads to the need for a method that secures food safety (CAC, 1997). Foods are considered safe when there is a low risk of harm to the consumer at the moment of consumption and, when it is prepared and/or eaten according to its rules of use (Mensah & Julien, 2011).

Hazard Analysis Critical Control Point (HACCP) approach is the internationally recognized strategy to ensure food safety. The Pillsbury Company, which collaborated with NASA and the U.S. Army laboratories initially developed it, in order to ensure that food sent in space programs were microbiologically safe. For this method to be effective, a deep understanding of processes and products, must exist since it is based on the identification of critical control points (CCP) throughout the production, distribution and

storage of food (Stringer, 2005, Stringer & Hall, 2007). CCP represent '*step at which control can be applied and is essential to prevent or eliminate a food safety hazard or reduce it to an acceptable level*' (CAC, 1997).

1.2 Food preservation

Pathogens present in raw materials may be partly or totally eliminated during processing or preparation. Therefore, there is a greater demand for new processing techniques that, at the same time, reduce the level of microbiological contamination and increase the level of safety of food, without compromising the desirable properties of food products. Currently, food preservation methods lead to the inactivation of microorganisms and enzymes and/or inhibition of microbial activity and growth (van Schothorst, *et al.*, 2009).

An ideal method of food preservation should fulfill some requirements: (i) extension of shelf life and safety by inactivating spoilage and pathogenic microorganisms; (ii) preservation of organoleptic and nutritional attributes of food; (iii) do not leave residues; (iv) inexpensive and convenient to apply; (v) not raise objections from consumers and legislators (Raso & Barbosa-Cánovas, 2003).

1.2.1 Thermal food processing

Traditional food processing methods frequently depend on the application of high temperatures. Processing using heat ensures safe food production and increased shelf time, once inactivate microorganisms and enzyme activity is reduced (Raso & Barbosa-Cánovas, 2003, Zhang, *et al.*, 2011). However, exists many factors that conditioned the extent of this impact, like properties of the microorganism, heat susceptibility of microbial strains and food chemical composition (Miller, *et al.*, 2009). Besides, this type of treatment may cause undesirable changes in food properties that affect nutritional and organoleptic attributes. Some of these changes are related to color and flavor given that many vitamins, as color

and flavor compounds, are degraded under heat treatments (San Martín, *et al.*, 2002, Morris, *et al.*, 2007, Zhang, *et al.*, 2011).

The two most common techniques used to process and preserve foods are thermal pasteurization and thermal sterilization (Zhang, *et al.*, 2011). The first technique is usually used for high-acid food products (pH <4.6), and may also be used for low-acid food products (pH > 4.6) followed by refrigeration. The second is the thermal treatment with best results when applied to low-acid food products, it uses temperatures around 121 °C for several minutes (Zhang, *et al.*, 2011).

Several attempts of optimization of thermal processing in order to obtain a maximum effectiveness against microorganisms, with minimal deterioration of the quality of food have been made in the last decades. For example, processes such as high-temperature short-time (HTST) pasteurization, that uses temperatures around 72 °C for 15 seconds, and ultra-high temperature (UHT) sterilization show less loss of vitamins in milk, compared to the conventional methods of pasteurization and sterilization, respectively. However, modern thermal technologies still cause changes in the texture and fresh flavor of processed foods (Lado & Yousef, 2002, Zhang, *et al.*, 2011).

1.2.2 Non-thermal food processing

Assumed the consumer demand for high-quality convenience food, particularly in terms of natural aroma and flavor, as well as the absence of additives and preservatives, there is a growing interest on innovative approaches to food processing (Zhang, *et al.*, 2011). Alternative technologies are occasionally designated as 'non-thermal' food processing. Non-thermal food processing technologies aim to exert a minimal impact on the nutritional, physic-chemical and sensory properties of food, at the same time that extends shelf life. They use temperatures below the temperature typically used in thermal processing so is expected a minimal degradation of food (Lado & Yousef, 2002, Raso & Barbosa-Cánovas, 2003, Devlieghere, *et al.*, 2004, Morris, *et al.*, 2007).

Pulsed electric fields (PEF), ionizing radiation, non-ionizing radiation and high pressure processing (HPP) are some of new non-thermal inactivation technologies investigated. PEF treatment consists on the delivery of pulses at high electric field intensity

(5-55 kV cm^{-1}) for a short duration (1 – 100 μs) to a food sited between two electrodes (Lado & Yousef, 2002, Mañas & Pagán, 2005). Ionizing radiation treatment consists on the application of gamma rays from cobalt-60, electron beams or X-rays to foods. Electron-beam technology is safer than gamma radiation, since this do not use radioactive isotopes (Lado & Yousef, 2002, Mañas & Pagán, 2005). Ultraviolet (UV) energy is a non-ionizing radiation that can inactivate microorganisms at wavelengths in the range of 200-280 nm (Lado & Yousef, 2002). HPP treatment consists on the application of pressures of 100 to 1000 MPa to food (Mañas & Pagán, 2005).

As a major advantage over thermal processes, non-thermal processing, and particularly HPP and irradiation, have the ability to inactivate/eliminate microorganisms at ambient, chilling and freezing temperatures (Lado & Yousef, 2002, Mañas & Pagán, 2005).

1.3 High pressure processing (HPP)

High pressure processing (HPP) has been considered as a valid alternative to conventional thermal pasteurization for the food preservation. Such confidence is due to its potential for inactivation of pathogenic and spoilage microorganisms and enzymes, while keeping food chemistry intact, with minimal effects on taste, texture, appearance, or nutritional value (San Martín, *et al.*, 2002, Yang, *et al.*, 2012).

Despite its use in the food areas has gained greater attention in the last decades, the use of this technology dates back to the late 19th century (table I). In 1899, high pressure was used to preserve milk, and subsequently extended to fruits and vegetables (Rastogi, *et al.*, 2007). Eighty years after these pioneer applications, Japan restarted the application of HPP in food processing in 1990, by marketing a line of HP-treated jams, jellies, and sauces packaged by Meidi-ya. From this date on, other countries followed the HPP to food processing (Trujillo, *et al.*, 2002, Rastogi, *et al.*, 2007).

Table I - History of HPP for food (adapted from San Martín, *et al.*, 2002).

Years	Events
1895	Royer (France) used high pressure to kill bacteria
1899	Hite (USA) used high pressure to preserve food
1980-1989	Japan started the production of fruit juices and jams using HPP
1990-1999	Avomex (USA) started the production of guacamole using HPP, from avocados with a shelf time prolonged and fresh flavor
2000	Europe began to produce and market fresh fruit juices (mostly citrus), and poultry. The USA began to sell fruit juices, poultry, and oysters with easy opening shell processed by HPP
2001	Approval of the sale of fruit juices, and fruit pieces processed by HPP in UK

In recent years, HPP has been shown to be commercially attractive given its advantages. Because of the possibility to process food at ambient temperature or colder, the instant transmittance of pressure through the system, the occurrence of microbial death by without the use of heat or additives and preservatives, the possibility to create ingredients with novel functional properties, and to conserve natural flavor and texture of foods, HPP is regarded with growing interest (Rastogi, *et al.*, 2007).

This technology in the food industry has been introduced gradually in several countries, and it is now possible to find various HPP products on the market (Figure 1) (Heinz & Buckow, 2009). HPP is used primarily in industries of vegetable and fruit processing, inactivating spoilage microorganisms and enzymes, while ensuring the organoleptic, sensory and nutritional qualities of these foods (Devlieghere, *et al.*, 2004, Rastogi, *et al.*, 2007). Some of the most common products are fruit jellies, sauces, rice, cakes and desserts, and guacamole (USA), being later packed after processing. HP-treated sliced ham (Spain), packed before processing has a shelf time 3 to 8 weeks extended in relation to the non-treated product (San Martín, *et al.*, 2002). Fruit juices (France and Portugal) and meat (Japan) are also being subjected to this method of processing (San Martín, *et al.*, 2002, Rastogi, *et al.*, 2007). The use of HPP in oysters brought advantages both in terms of the inactivation of microorganisms, and in the process of shell opening. Oysters are preferentially consumed raw and are sometimes associated with outbreaks of gastroenteritis caused by *Vibrio spp.*. The use of HPP increased the microbiological quality

and facilitated the opening of the bivalves, without any loss in terms of flavor or texture (San Martín, *et al.*, 2002, Rastogi, *et al.*, 2007). In the processing of dairy products, HPP has been used in milk-clotting processes. HPP of milk for cheese production reduce the time of rennet formation, and accelerates cheese production by reducing the ripening time. When used in animal products such as meat, HPP induces changes in muscle enzymes and the proteolysis rates, leading to an improvement of its texture and structure (Devlieghere, *et al.*, 2004, Rastogi, *et al.*, 2007).

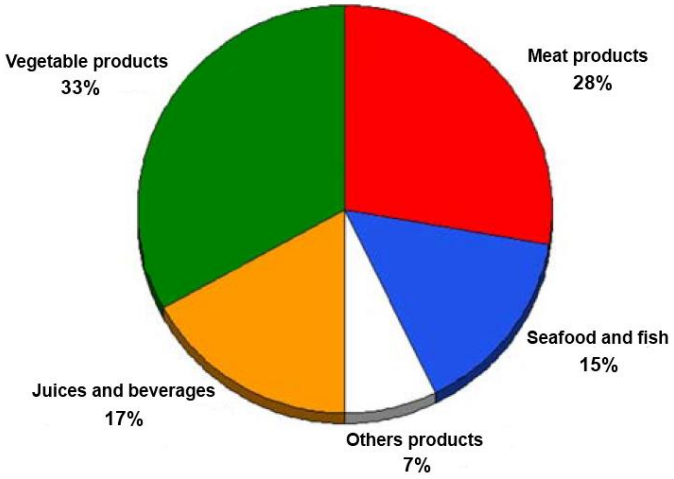

Figure 1 - Different sectors of the food industry in which is used HPP preservation (extracted from Heinz & Buckow, 2009).

1.3.1 General principles of HPP

HPP can be classified as a cold pasteurization technique, a non-thermal food preservation method that uses pressure – 'force per unit area applied on a surface in a direction perpendicular to this surface' (Rivalain, *et al.*, 2010) – to inactivate pathogens and vegetative forms of spoilage microorganisms. At the same time, it increases shelf life and retains the original features of food (Morris, *et al.*, 2007). This technique can be used in different types of food matrices, solid or liquid, to pressure values between 100 and 1000 MPa (1 MPa = 9.87 atm = 10 bar = 145 psi) in a range of temperature between -20 and 80 °C, and in variables times of application, from a few seconds to more than 20 minutes (San Martín, *et al.*, 2002, Morris, *et al.*, 2007).

There are two basic principles that govern HPP in the food industry: *Le Chatelier's Principle* and *Isostatic Principle*. During the pressurization, a decrease in food volume occurs, being this reduction proportional to the pressure applied, and the food material returns to its initial volume during decompression (Patterson, Ledward, & Rogers, 2006). This effect follows *Le Chatelier's Principle*, in which applying pressure promotes the state of smaller volume, and accelerates processes that lead to transition state that assume a smaller volume compared to the ground state (Oger & Jebbar, 2010). Besides that, pressure is applied in an isostatic mode, i.e. the transmission of pressure occurs uniformly and almost instantly through the food material regardless of its shape and size, providing that it does not deform when subject to such conditions and that it returns to the original shape. This feature makes this technique suitable for the inactivation of pathogens that are either surface located, or imbed in the food matrix (Rastogi, *et al.*, 2007, Neetoo, *et al.*, 2011, de Alba, *et al.*, 2012).

1.3.2 HPP Equipment

Processing systems for high pressure treatment of food products are essentially formed by a pressurization vessel (Figure 2), a system of pressurization, heating/cooling systems and product handling devices (Devlieghere, *et al.*, 2004).

The industrial equipment used in HPP can be discontinuous for solid, viscous and particulated foods (batch processing), and semi-continuous for liquid foods (bulk processing) (Devlieghere, *et al.*, 2004).

Batch processing is the most common process in the food industry. In batch treatments, the foods are pre-packaged and treated in a chamber surrounded by pressure-transmitting fluid. HPP of solid foods starts with the removal of air from the food. This initial procedure is essential to ensure that the work of compression is not wasted in the air in the system. A typical process consists of loading the vessel with food to be processed, and filling the remaining space of the vessel with pressure-transmitting fluid. The vessel is closed and the process of pressurization begins by adding more pressure-transmitting fluid by an intensifier until it reaches the desired pressure. After treatment, the vessel is decompressed by releasing the fluid (Balasubramaniam, *et al.*, 2008). There are several

pressure-transmitting fluids that can be used in laboratory pressure equipment, namely water, castor oil, silicone oil, glycol-water mixtures, and sodium benzonate solutions (Zhang, *et al.*, 2011). HPP of liquid foods is identical to the one used to process solid foods (Balasubramaniam, *et al.*, 2008).

In bulk processing, the foods are submitted to pressure before packaging and are aseptically packaged after pressurization (Balasubramaniam, *et al.*, 2008). Exists two or more pressure vessels in the equipment used for this processing method in order to compress food. Initially, the pressure vessel is filled with liquid food to be processed. Afterwards, the inlet valve is closed and the compression of food starts through the introduction of pressure-transmitting fluid behind a free piston. After treatment the vessel is decompressed and the treated liquid food can be transferred to sterile containers (Balasubramaniam, *et al.*, 2008, Zhang, *et al.*, 2011).

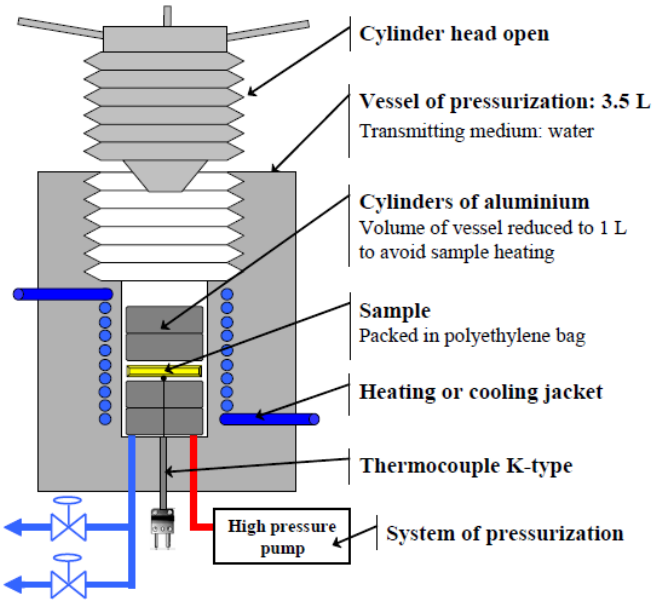

Figure 2 - Scheme of the high pressure vessel from ACB pressure systems (extracted from Chapleau, *et al.*, 2006).

1.3.3 Factor involved in the efficiency of HPP

HPP technology can be used for different treatments, such as food pasteurization, sterilization, blanching, freezing and defrosting, depending on the chosen conditions

(Figure 3), i.e. the combination of pressure with temperature and/or atmosphere (Zhang, *et al.*, 2011).

Pasteurization by HPP inactivates spoilage and pathogenic bacteria, yeasts, and molds, but it presents a limited effectiveness against spores and enzymes (Zhang, *et al.*, 2011). The level of bacterial inactivation depends on the type of microorganism, food matrix composition, and pH, being necessary to choose the appropriate combined treatment to obtain the best results of microbial inactivation and increased shelf life (Raso & Barbosa-Cánovas, 2003, Zhang, *et al.*, 2011).

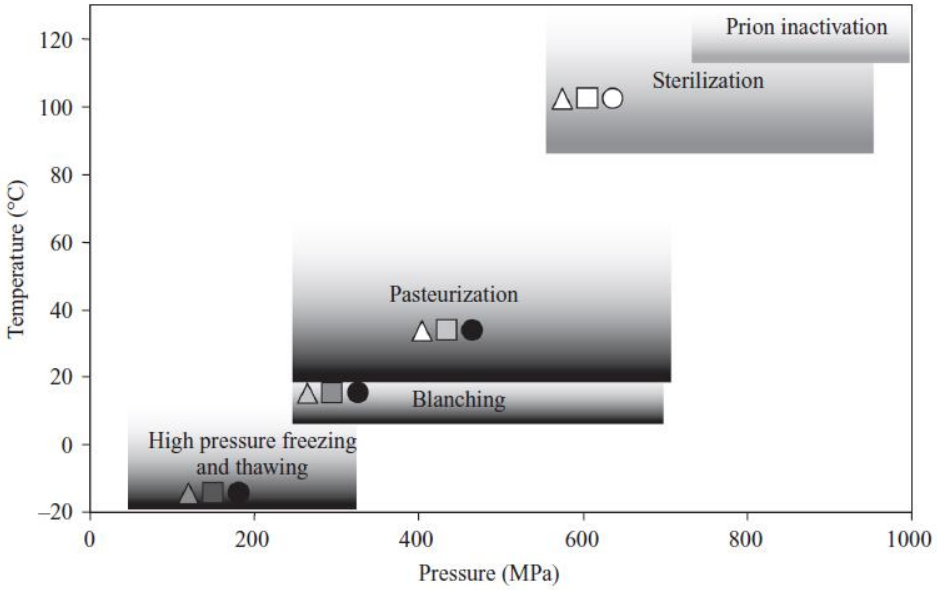

Figure 3 - Different processing effects given the pressure-temperature conditions chosen. Filled symbols represent no effect, and open symbols represent inactivation. Vegetative bacteria, yeast, and mold (□), bacterial spores (○), and enzymes (Δ) (extracted from Zhang, *et al.*, 2011).

1.3.3.1 Food matrix

The composition of the food substrate can affect the level of inactivation of microorganisms by HPP. The implementation of this method can inactivate microorganisms, or modify the properties of food, being essential to consider both the effects during the optimization of processing conditions (Doona & Feeherry, 2007). For example, the reduction of *E. coli* O157:H7 and *L. monocytogenes* is different when subject

to the same conditions of processing, but in different substrates. In both cases it was observed a greater resistance to pressure when the organisms were treated in UHT milk than in buffer solution or poultry meat (Patterson, *et al.*, 1995). Microbial inactivation can also be conditioned by the pH and water activity (a_w) of food, and the results presented by different authors are contradictory. In general, microorganisms are more sensitive to pressure in lower pH environments, and pressure-damaged cells survive less in acidic environments. This represents an opportunity for the HPP treatment of fruit juices, in that even microorganisms survive the pressure treatment, for example *E. coli* O157:H7, they are damaged and die during cold storage due to the characteristics of the environment, i.e. acid conditions (Linton, *et al.*, 1999). However, contrary results have been obtained, and different strains of *Enterobacteriaceae* are less sensitive to HPP inactivation in low pH environments (Raso & Barbosa-Cánovas, 2003, Doona & Feeherry, 2007).

1.3.3.2 Temperature

HPP can be combined with temperature for best results. Vegetative cells of bacteria have a greater resistance to pressure at temperatures between 20 and 30 °C. Resistance decreases when the pressure is combined with heat, even at non-lethal temperatures. This combination allows a considerable inactivation, over 6 log, to lower pressures, and the variation in pressure resistance among the strains is lower than that observed in the combination of HPP with room temperature. Vegetative cells of yeasts and molds, however, are very sensitive to treatments by HPP at room temperature (Raso & Barbosa-Cánovas, 2003).

During treatment, compression and decompression may result in a transient temperature change in the product to be processed (Balasubramaniam, *et al.*, 2008). There may be a variable increase in the temperature of the product, depending on the composition of the product substrate. Generally, the application of high temperatures leads to an increase of approximately 3 °C per 100 MPa (Morris, *et al.*, 2007, Rastogi, *et al.*, 2007). However, this increase can be as high as 8-9 °C per 100 MPa, in foods with significant amounts of fat, such as butter and cream. During decompression these cooled resuming its initial temperature (Rastogi, *et al.*, 2007).

1.3.3.3 Carbon dioxide

Carbon dioxide (CO_2) has antimicrobial effect that increase when is applied under pressure. The combination of HPP with CO_2 was tested in natural flora and spoilage as well as in pathogenic microorganisms, but others parameters such as time, temperature, a_w, and pH, have to be considered in this combination. An increase in temperature and/or pressure combined with a decrease in pH improves the antimicrobial effect. However, a low a_w decreases the inactivation efficiency (Ballestra & Cuq, 1998).

There are different suggestions for the mechanism behind microbial inactivation using CO_2, namely, the quick release following compression and the transference of a larger number of molecules of CO_2, during pressurization, through the membrane reducing de internal pH, which can affect key enzymes (Nakamura, *et al.*, 1994, Garcia-Gonzalez, *et al.*, 2007).

1.3.3.4 Antimicrobials

Given the consumer demand for food free of additives and preservatives, food industry searches for natural alternatives. Several natural antimicrobial compounds produced by animals, plants and microorganisms, have been investigated. Examples of these compounds are lactoperoxidase present in milk, lysozyme present in egg white and figs, saponins and flavonoids present in herbs and spices, bacteriocins produced by lactic acid bacteria (LAB), and chitosan from shrimp shells (Devlieghere, *et al.*, 2004). Most of natural antimicrobial compounds have a restricted antimicrobial spectrum, directed towards a small group of microorganisms, and often restricted to Gram-positive bacteria (García-Graells, *et al.*, 2000).

Bacteriocins represent a huge and diverse group of ribosomally synthetized extracellular antimicrobial proteins or peptides with bacteriostatic or bactericidal effect. The most studied bacteriocins are nisin (*Lactococcus lactis*), pediocin (*Pediococcus acidilactici*), and sakacins (*Lactobacillus sakei*) (Devlieghere, *et al.*, 2004). Nisin was recognized as a food preservative by FAO/WHO in 1969 (FAO/WHO, 1969), and is the

only preservative approved by the Food and Drug Administration (FDA) for use in the food industry. These group of natural antimicrobial compounds are being combined with non-thermal methods, such as HPP, to increase food safety, once HPP by itself may not be a safe pasteurization process due to development of high levels of baroresistance in certain vegetative cells of bacteria (Gallo, *et al.*, 2007).

1.3.3.5 Pressurization and depressurization kinetic

The influence of different pressurization and depressurization rates on inactivation of several microorganisms has been studied and discussed, but contradictory results and conclusions persist (Hayakawa, et al., 1998, Smelt, 1998, Rademacher, et al., 2002). Some authors assumed that a low compression rate induces a stress response from microbial cells, and consequently leads to a lesser effective process, i.e. a lower microbial inactivation (Smelt, 1998). Complementary, a high decompression rate might induce a fast adiabatic expansion of water generating an impulsive force that combining with pressurization would result in higher inactivation effect than pressurization alone (Hayakawa, et al., 1998, Noma, et al., 2002). Also, yeasts would be more sensitive to fast depressurization than vegetative bacteria, because of the existence of vacuoles in their cells (Smelt, 1998).

The effect of pressurization and depressurization rates on inactivation of *Salmonella typhimurium* and *Listeria monocytogenes* (Chapleau, *et al.*, 2006), *Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus* and *S. typhimurium* (Noma, *et al.*, 2002), *Bacillus stearothermophilus* spores (Hayakawa, *et al.*, 1998), and *Listeria innocua* (Rademacher, *et al.*, 2002) has been studied. Chapleau, *et al.* (2006) observed the most significant reduction of microorganisms when they were exposed to a fastest pressurization/depressurization kinetic parameters. Hayakawa, *et al.* (1998) and Noma, *et al.* (2002) described that fast depressurization was more effective than slow depressurization treatment in inactivating bacterial spores and vegetative bacteria, respectively. In your turn, Rademacher, *et al.* (2002) do not find statistically significant differences on inactivation of *L. innocua* using two processes, namely fast pressurization

(500 MPa min^{-1}) and slow depressurization (100 MPa min^{-1}), and the reverse, i.e. slow pressurization (100 MPa min^{-1}) and fast depressurization (500 MPa min^{-1}).

1.4 Effects of HPP on microorganisms

When applied at room temperature, HPP can inactivate vegetative cells and certain enzymes. However, the responses to pressure differ from microorganism to microorganism. Generally, it is easier inactivate organisms more complex and with larger cell size (Balasubramaniam, *et al.*, 2008). The less resistance microorganisms are vegetative bacterial cells, followed by yeasts and molds, viruses, and finally bacterial endospores, which are the most resistant structures (Doona & Feeherry, 2007, Balasubramaniam, *et al.*, 2008).

Depending on the composition of the cell wall and the growth phase of vegetative cells, the resistance to pressure may vary considerably. Gram-positive bacteria are more resistant than Gram-negative cells, and in stationary phase, cells are more resistant than in exponential phase (Doona & Feeherry, 2007, Zhang, *et al.*, 2011). In stationary phase bacteria can synthesize proteins that confer them protection against adverse conditions such as oxidative stress, high salt concentrations, and high temperature (Doona & Feeherry, 2007).

In studies with pathogenic microorganisms, such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhimurium*, large differences in results have been observed, with reductions from 0.5 to 8.5 log units. Besides, differences between strains belonging to the same genus or specie have been reported too (Rendueles, *et al.*, 2011). This variability may be related with differences in growth phase.

Many studies have attempted to enlighten the mechanisms involved in the inactivation and death of vegetative bacterial cells by HPP. It is known that this technique can damage membranes, affect homeostasis, alter the morphology of the cell, and denature and inactivate proteins (Zhang, *et al.*, 2011). Cell membrane is considered the first site of damage in bacteria inactivated by pressure. This damage was confirmed by scanning electron microscopy (SEM) observations, in which bud scars on surface of pressurizes cells have been observed (Rivalain, *et al.*, 2010). Through SEM it was also possible to

observe an increase in average cell area and volume in *Staphylococcus aureus* and *Escherichia coli*. These alterations can be justified by modifications of membrane properties, such as denaturation of membrane-bound proteins, decrease in membrane potential and cellular ATP content, and/or phase transition of the lipid bilayer of membrane (Pilavtepe-Çelik, *et al.*, 2008, Rivalain, *et al.*, 2010).

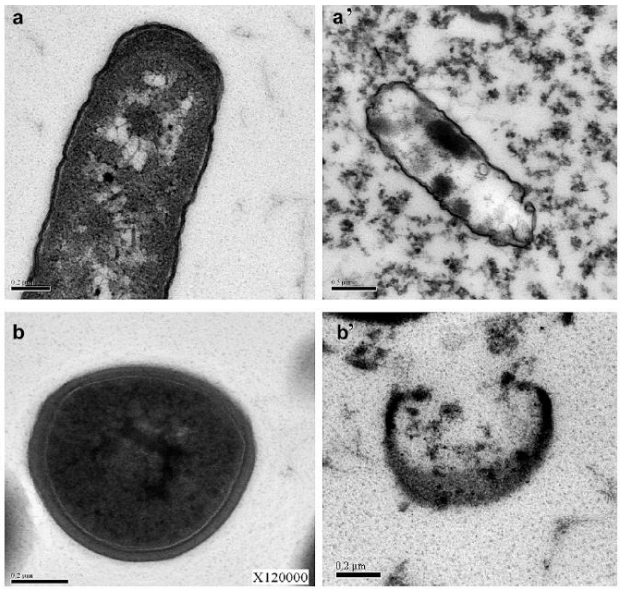

Figure 4 – Cell structures of untreated *E. coli* (a) and *S. aureus* (b), and 500 MPa for 30 min treated *E. coli* (a') and *S. aureus* (b') under TEM (extracted from Yang, *et al.* (2012).

Transmission electron microscopy (TEM) images confirmed morphological modifications in *S. aureus* and *E. coli* (Figure 4). When cells were treated with low pressure, they keep distinct membrane and cell wall, but when submitted to high pressure (500 MPa) breakdown of the peptidoglycan layer and an aggregation of cytoplasmatic material occur (Yang, *et al.*, 2012).

Most yeasts and molds are relatively sensitive to pressure, being inactivated when exposed to 300-400 MPa at room temperature, but ascospores are more resistant, requiring treatments at higher pressures (Balasubramaniam, *et al.*, 2008). Some authors have compared ascospores with vegetative cells, and observed that the former were found to be 5 to 8 times more resistant to pressure. Besides, it was shown that HPP instead of inactivating, can activate and induce germination of ascospores (Zhang, *et al.*, 2011).

Viruses can present a wide range of pressure resistance but can generally be inactivated in the same conditions as those used to inactivate bacteria (Balasubramaniam, *et al.*, 2008). Some human viruses are relatively sensitive to pressure, while poliovirus and picornavirus are very resistant, being inactivated by less than 1 log at 600 MPa during 1 h. Others, such as feline calicivirus, are completely inactivated at 275 MPa during 5 minutes. Inactivation of these microorganisms seems to be more dependent on the pressure applied than on the time during which it is applied (Balasubramaniam, *et al.*, 2008, Zhang, *et al.*, 2011). Usually, naked viruses are more resistant to the pressure than enveloped viruses. Depending on the pressure, HPP can denature capsid proteins in an irreversible or reversible way or damage the envelope, while keeping viral nucleic acids intact. These injuries are responsible by incapacity of viruses for cell binding and infection initiation (Rendueles, *et al.*, 2011, Zhang, *et al.*, 2011).

Bacterial spores can be extremely resistant to pressure, requiring more aggressive conditions than vegetative cells, namely higher pressure, higher temperature, and longer holding times. Some endospores can be resistant to pressures above 1000 MPa at room temperatures. Among pathogenic spores, *Clostridium botulinum* is the most resistant to pressure and for nonpathogenic species, *Bacillus amyloliquefaciens* produces the most resistant endospores (Balasubramaniam, *et al.*, 2008). However, at low pressure (60 to 100 MPa) HPP can induce spore germination, being this process temperature dependent. This is due to electrostriction, this implicates a decreased volume affected by the high electric field of the ion that influence the orientation of water molecules causing a collapse of the water structure, that allows core hydration, triggering the germination process. After that, spores can be inactivated at higher pressures (San Martín, *et al.*, 2002). It has been demonstrated that the sensitivity of germinated spores to subsequent stresses is affected by the pressure used to induce germination. For example, *Bacilus subtilis* spores were more sensitive to pressure inactivation, and others treatments, such us UV light and hydrogen peroxide, when spores germinated at 100 MPa comparatively with spores germinated at 500 MPa (Wuytack, *et al.*, 1998).

To inactivate spores directly, HPP has been combined with several antimicrobials, such as bacteriocins, sucrose laurate, and citric acid (Zhang, *et al.*, 2011).

1.5 Recovery after HPP

HPP is a technique already used for food processing. However, as important as understanding the mechanisms involved in the inactivation of microorganisms is to know if after being subjected to high pressures, microorganisms can recover viability and grow during storage.

Some authors suggest that microorganisms suffer sublethal damage that can be at the level of i) cytoplasmatic membrane – structural damage or, ii) intracellular components – physiological damage. These damages can be reversed if the cells are stored in conditions advantageous to recovery and growth (Cheftel & Culioli, 1997, Bull, *et al.*, 2005, Han, *et al.*, 2011). The storage temperature can influence the inactivation/recover of microorganisms, as well as the temperature of growth before processing and the media used in the recovery (Bull, *et al.*, 2005).

L. monocytogenes fails to recover during storage in milk at 4 and 30 °C (Bull, *et al.*, 2005), but on the contrary, when stored at 15 °C, a full recovery occurs after 14 days. Low levels of recovery have been observed for *L. innocua* and psychrotropic bacteria during long chilling times (30, 60 or 120 days) (Yuste, *et al.*, 1999, Garriga, *et al.*, 2004). These results go against the observed in previous studies (Yuste, *et al.*, 1998), in which a loss in ability to grow at low temperatures by psychrotrophics microorganisms, as is the case of *L. monocytogenes* was observed. In other studies, a decreased of pH during the storage at chilling temperatures was detected, suggesting that the inability to recover during storage at chilling temperatures are related with a low pH (Han, *et al.*, 2011).

1.6 *Listeria innocua*

The genus *Listeria* is composed of short rods with 0.4–0.5 μm of diameter and 1.2 μm of length, usually appear singly or in short chains. Can be classified like Gram-positive bacteria, however, with time cells can lose their ability to retain stain. Belonging to the family *Listeriaceae*, this genus is closely related to the genera *Bacillus, Clostridium, Enterococcus, Streptococcus* and *Staphylococcus*. *Listeria spp*. are facultative anaerobic bacteria with no capsule, low GC content, and do not produce spores (Hain, *et al.*, 2006,

Hain, *et al.*, 2007, Nufer, *et al.*, 2007). These microorganisms grow at temperatures between 0 and 45 °C, but their optimum temperature for growth is between 30 and 37 °C (Nufer, *et al.*, 2007). Cells are motile at 10-25 °C due to the presence of two or six peritrichous flagella that gives them a characteristic movement – tumbling motility. The absence of flagella at 37 °C is essential for a full virulence, in case of pathogenic species like *L. monocytogenes* (Vos, *et al.*, 2009). These bacteria have the ability to grow in high concentrations of salt (10% NaCl) and at pH values between 4.5 and 9. This genus entails six species: *L.monocytogenes*, *L.ivanovii*, *L.innocua*, *L.grayi*, *L.welshimeri* and *L.seeligeri*. Only the first two species are pathogenic. *L.monocytogenes* causes opportunistic infections in humans and animals, and *L.ivanovii* is essentially responsible for infections in animals (Vázquez-Boland, *et al.*, 2001, Hain, *et al.*, 2006).

Listeria can be isolated from several environmental sources, in particular soil, water, plants, feces, meat, fish, dairy products and decaying plant matter, being decaying plant matter their natural habitat and, at the same time, the vehicle of transmission to the vertebrate host (Vázquez-Boland, *et al.*, 2001, Hain, *et al.*, 2006). Given the ubiquity of the genus, it can also be found in facilities and equipments involved in the processing and storage of foods, which gives them a great importance on public health (Hain, *et al.*, 2006, Nufer, *et al.*, 2007). Some strains are able to survive for long periods under hostile conditions and persist in food processing equipments, due to its ability to form biofilms on several surfaces (Todd & Notermans, 2011).

L. innocua, is a non-pathogenic species that has been often used as a surrogate for the pathogenic *L. monocytogenes* in biological studies, since presents similar responses to chemical or thermal treatments, and occurs in the same natural environments (Gleeson & O'Beirne, 2005, Gallo, *et al.*, 2007, Miller, *et al.*, 2009).

L. monocytogenes is a food pathogen responsible for an opportunistic infection called listeriosis (Nufer, *et al.*, 2007). Several food products have been involved in outbreaks caused by this pathogen, a major source is raw material (Todd & Notermans, 2011). Soft cheese, fruits and vegetables, and cooked meat were considered of special risk. However, there are others products that are considered of low risk and that have been connected to listeriosis transmission, such as corn (Aguado, *et al.*, 2004, Gleeson & O'Beirne, 2005). In short, listeriosis outbreaks are caused by consumption of ready-to-eat dairy and meat products (Bull, *et al.*, 2005).

The infections by *Listeria* present several clinical symptoms as meningitis, meningoencephalitis, septicaemia, abortion, prenatal infection and gastroenteritis (Hain, *et al.*, 2007). Human listeriosis is caused, in about 99%, by the consumption of contaminated foods. Despite an occurrence as low as 2-15 cases per million population per year, the mortality is high, about 20-30%, in infected patients, especially in pregnant women, elderly and immunocompromised patients (Hain, *et al.*, 2006, Hain, *et al.*, 2007, Carvalheira, *et al.*, 2010).

1.7 Objectives

The main purpose of this work was assess the importance of the rate of pressurization as a parameter determining the efficiency of inactivation of *Listeria innocua* by HPP, in order to contribute to the definition of efficient inactivation protocols. For that, *Listeria innocua* was submitted to slow, intermediate, and fast pressurization, followed by a constant pressure – 300 and 400 MPa-, during a pre-established holding time – 1 and 5 minutes. In addition, recovery after pressurization at the conditions for which best inactivation results were achieved, was also evaluated.

2. Material and methods

2.1 Bacterial strain

For this study a strain of *Listeria innocua* (10528, National Collection of Type Cultures, UK; NCTC, courtesy of *Escola Superior de Biotecnologia da Universidade Católica Portuguesa, Porto*) was used. The initial culture was inoculated in Tryptic Soy Agar (TSA, Liofilchem®) and incubated at 37 ºC for 48 h, in order to be obtained isolated colonies for later use in the preparation of new cultures in liquid medium. The cultures were maintained on TSA plates and stored at 4 ºC, and streaked monthly from these stocks. An isolated colony was also inoculated in 50 mL of Tryptic Soy Broth (TSB, Liofilchem®) and incubated overnight at 37 ºC with continuous agitation at 170 rpm in an orbital shaker (Optic Ivymen System), for subsequent cryopreservation in 20% sterile glycerol and storage at -80 ºC.

2.2 Characterization of growth kinetics in liquid medium and cellular viability tests

The characterization of growth kinetics in liquid medium was performed in order to allow the standardization of experimental procedure to adopt in the high pressure inactivation experiments. This procedure made it possible to ensure that cultures subject to HPP were in equivalent growth phase (stationary phase). In order to characterize growth in the experimental conditions adopted, the growth curve was constructed.

An isolated from a TSA plate was inoculated in TSB, and incubated during 20 h at 37 ºC with continuous agitation at 170 rpm. After incubation, 50 µL of culture was transferred to 50 mL of TSB previously placed to 37 ºC, allowing the culture to adapt more quickly to the new environment and accelerating growth, and incubated in the same conditions. For the characterization of growth kinetics, 200 µL of later culture was transferred to 200 mL of TSB previously placed to 37 ºC. This fresh culture was incubated in the same conditions of the previous. Bacterial growth was evaluated by the optical density at 600 nm (OD_{600}). For that, 2 mL aliquots were collected hourly and transferred to disposable cells (VWR®) for the measurement of OD_{600}, on a spectrophotometer (Dynamica Halo DB-20 UV-Vis Double Beam Spectrophotometer), using non-inoculated

TSB as blank. Samples with OD_{600}>1 were diluted with TSB (1:1) and the readings were corrected for the dilution factor.

In parallel with OD_{600} readings, an aliquot of the culture was serially diluted in Ringer solution (Merck Millipore) and pour-plated in duplicate in TSA. Plates were incubated for 48 h at 37 °C and colonies were counted in the plates of the most suitable dilution. The values of the two replicates were averaged and corrected for the dilution factor for the calculation of the concentration of colony forming-units (CFU mL^{-1}).

Three independent growth curves were constructed in the same experimental conditions and the values were averaged for the graphic representation of the growth curve.

2.3 High pressure processing (HPP)

2.3.1 Culture conditions and preparation of inocula

For each high pressure experiment, an isolated single colony was inoculated in 50 mL of TSB, incubated during 20 h at 37 °C with continuous agitation at 170 rpm. From this culture, an aliquot of 200 µL of culture was transferred to 200 mL of TSB previously placed to 37 °C. This second culture was incubated in the same conditions of the first during the time estimated as corresponding to stationary phase (12 h), as inferred from the growth curves previously constructed.

2.3.2 Characterization of inactivation kinetics by HPP

In order to set out the most adequate conditions of pressurization for the strain of *L. innocua* used in this study, different values of pressure (100 MPa, 200 MPa, 300 MPa, 400 MPa, 500 MPa, and 600 MPa) were applied during 5 minutes at room temperature. The results of these assays were used to elect the most suitable pressure for further inactivation experiments.

Before the treatments, OD_{600} of the culture confirmed that the bacterial culture was in the same growth phase ($OD_{600} \approx 1.6$), and aliquots of 100 µL were taken for the

determination of concentration of viable cells in the culture (N_0). For that, the culture aliquots were serially diluted in Ringer solution (Merck Millipore) and pour-plated in TSA, in triplicate. The plates were incubated for 48 h at 37 °C and colonies were counted in the replicates of the most suitable dilution. The concentration of viable cells (CFU mL^{-1}) was determined from the average of the replicates, corrected for the dilution factor.

Meanwhile, the culture was maintained in melting ice, and distributed in pressurization microtubes (Microtube PE 0.5 mL Beckmann), previously sterilized with bleach, ethanol, and ultraviolet irradiation (UV). The microtubes were completely filled and hermitically capped, in order to avoid air bubbles. The microtubes were arranged in groups of 3, covered in parafilm (Parafilm®) and put into plastic bags that were filled with sterile water, sealed, and kept in melting ice until 10 minutes before pressurization.

For each experimental condition, two plastic bags, with three microtubes each, were loaded into the pressurization vessel of high pressure equipment (Unipress Equipment, Model U33, Poland) (Figure 5), and submitted to pressure. The pressure holding time reported did not include the pressurization and depressurization times. For these initial experiments, the pressurization rate was set to the medium value as well as depressurization rate.

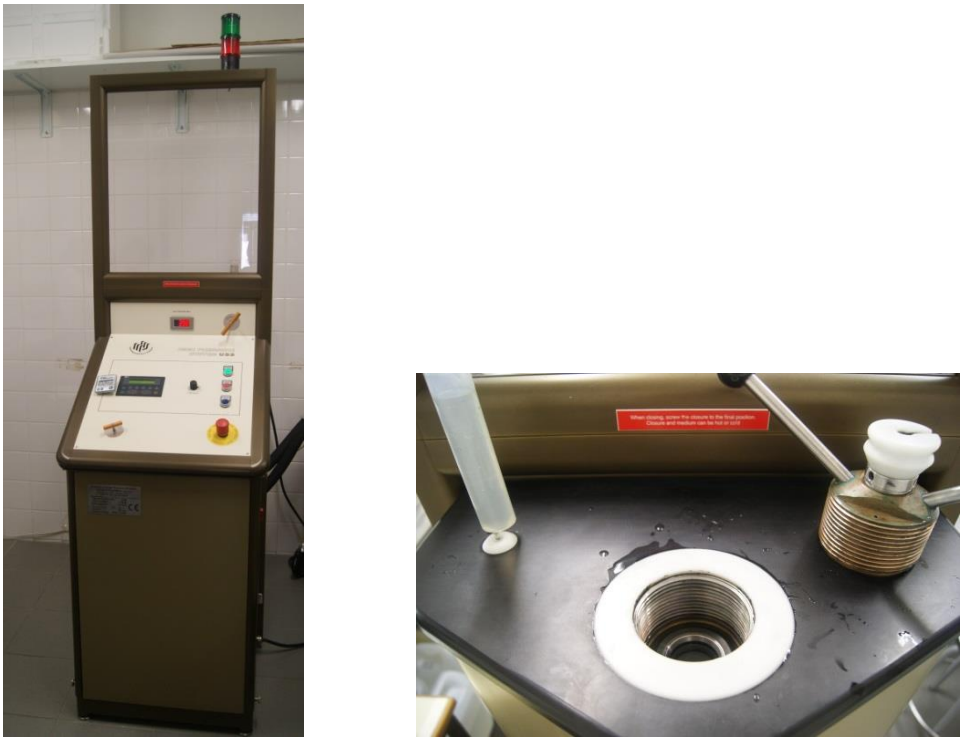

Figure 5 – A) High pressure equipment (Unipress Equipment, Model U33, Poland). B) Detail of the pressurization vessel.

The high pressure equipment used for this work is a 2006 model with a 35 mm diameter and 100 mm height pressurization vessel with a maximum capacity of 100 mL. It can achieve pressure up to 700 MPa, at a temperature range between -20 °C and 100 °C. Besides allows the application of different pressurization and depressurization rates, yet the choice of these rates cannot be done digitally, i.e. it is possible to control them manually but they must be calculated.

The pressure transmitting fluid was a mixture of 60% water and 40% propylene glycol (DOWCAL™, Dow). This fluid has an antifreeze action, which inhibits the formation of ice when making pressurizations at lower temperatures and during depressurization when temperature can drop to negative values.

After pressurization, the bags were kept in melting ice until processing of the samples. Cell suspension from each pressurized microtube was serially diluted in Ringer solution (Merck Millipore), and each dilution (from each sample/microtube) was pour-plated in TSA, in triplicate. The plates were incubated for 48 h at 37 °C and colonies were counted in the replicates of the most suitable dilution. The concentration of viable cells (CFU mL^{-1}) was determined from the average of the replicates, corrected for the dilution factor.

The inactivation factor (IF) was calculated as the logarithmic reduction of the concentration of viable cells using the expression $IF=\log_{10}(N_0/N_i)$, in which N_0 represents CFU mL^{-1} in the initial culture (untreated control) and N_i represent the value of CFU mL^{-1} after HPP. Three independent assays were conducted for each experimental condition and the average IF was calculated for graphic representation.

2.3.3 Inactivation by HPP with different pressurization rates and holding times

In order to evaluate the influence of pressurization and depressurization rates in the efficiency of inactivation of *L. innocua*, bacterial cells were submitted to slow, intermediate, and fast pressurization and depressurization. Two different values of pressure, 300 MPa and 400 MPa, were applied during 1 or 5 minutes at room temperature.

The OD_{600} of the stationary phase culture was checked and aliquots of 100 µL were taken for the determination of concentration of viable cells in the culture (N_0). For that, the

culture aliquots were serially diluted in Ringer solution (Merck Millipore) and pour-plated in TSA, in triplicate. The plates were incubated for 48 h at 37 °C and colonies were counted in the replicates of the most suitable dilution. The concentration of viable cells (CFU mL^{-1}) was determined from the average of the replicates, corrected for the dilution factor.

Meanwhile, the culture was maintained in melting ice, and distributed in pressurization microtubes (Microtube PE 0.5 mL Beckmann), previously sterilized with ultraviolet irradiation (UV). The sterilization method of microtubes has been changed comparing with the procedure used in characterization of inactivation kinetics by HPP. This change due to the possibility of the microtubes still contain ethanol when the culture is transferred to them, what affect the results, since ethanol leads to cell death. The microtubes were completely filled and hermitically capped, in order to avoid air bubbles. The microtubes were arranged in groups of 3, covered in parafilm (Parafilm®) and put into plastic bags that were filled with sterile water, sealed, and kept in melting ice until 10 minutes before pressurization.

For each experimental condition, two plastic bags, with three microtubes each, were loaded into the pressurization vessel of high pressure equipment and submitted to pressure. In these experiments, three different pressurization and depressurization rates were tested. They were calculated by dividing constant pressure value to be tested by the time it took to reach that pressure (pressurization rate), and to release the pressure (depressurization rate), being the rate given by MPa s^{-1}.

The concentration of viable cells in treated samples was determined like in untreated cells, and the IF associated to each experimental condition was calculated as previously described. Three independent assays were conducted and the average was calculated for graphic representation.

2.3.4 Recovery of viability after HPP

In order to check a possible recovery of *Listeria innocua* after being pressurized, cells were treated in the conditions that cause the highest sublethal effect (400 MPa) and lethal effect (500 MPa), during 5 minutes at room temperature.

Before the treatments, OD_{600} of the culture was confirmed that the bacterial culture was in the same growth phase, and aliquots of 100 µL were taken for the determination of concentration of viable cells in the culture (N_0). For that, the culture aliquots were serially diluted in Ringer solution (Merck Millipore) and pour-plated in TSA, in triplicate. The plates were incubated for 48 h at 37 °C and colonies were counted in the replicates of the most suitable dilution. The concentration of viable cells (CFU mL^{-1}) was determined from the average of the replicates, corrected for the dilution factor.

Meanwhile, the culture was maintained in melting ice, and distributed in pressurization microtubes (Microtube PE 0.5 mL Beckmann), previously sterilized with ultraviolet irradiation (UV). The microtubes were completely filled and hermitically capped, in order to avoid air bubbles. The microtubes were arranged in groups of 3, covered in parafilm (Parafilm®) and put into plastic bags that were filled sterile water, sealed, and kept in melting ice until 10 minutes before pressurization.

Two plastic bags, with three microtubes each, were loaded into the pressurization vessel of high pressure equipment and submitted to conditions that satisfied the purpose of these assays. This step was replicated. After pressurization, the bags were transferred to melting ice until processing.

A composite treated sample was obtained by combining in one single tube, the content of twelve microtubes. From the composite sample, an aliquot of 100 µL was taken for the determination of the concentration of viable cells (CFU mL^{-1}) as previously described. The remaining of the composite-sample was equally distributed by 6 microtubes that were stored at 4 °C (3 microtubes) or at 25 °C (3 microtubes) when value of pressure tested was 400 MPa. When pressurization was performed at 500 MPa a composite treated sample was obtained by combining in one single tube, the content of six microtubes. From the composite sample, an aliquot of 100 µL was taken for the determination of the concentration of viable cells (CFU mL^{-1}). The remaining of the composite-sample was equally distributed by 3 microtubes that were stored at 25 °C.

After 1, 5 and 10 days, the concentration of viable cells (CFU mL^{-1}) was determined as previously described. Cell suspension stored at 4 °C and 25 °C were serially diluted in Ringer solution and pour-plated in TSA, in triplicate, being each microtube a replicate. All the plates were incubated for 48 h at 37 °C before colony enumeration. CFU mL^{-1} was determined from the average of the replicates, considering dilution factor.

Inactivation factor (IF) was calculated as previously described. Three independent assays were conducted for each experimental condition and the average IF was calculated for graphic representation.

2.3.5 Statistical analysis

Normality was checked by the Shapiro-Wilk test and homogeneity of variances by the Levene test. When these assumptions were confirmed ANOVA was performed to assess the differences between tested conditions, and whenever significant differences were detected, the Tukey's post-hoc test was applied. When normality was not observed, the significance of the differences was evaluated by Kruskal-Wallis test. The level of significance was set to 0.05. All statistical analyses were conducted with the IBM SPPS Statistics 20.

3. Results

3.1 Growth kinetics in liquid medium

The growth curve of *Listeria innocua* in experimental conditions used in this work (cultivation in TSB and incubation at 37 °C with orbital agitation at 170 rpm) is represented in figure 6. Lag phase was undistinguishable, and exponential phase occurred for 8-9 hours. Stationary phase was generally reached within 9 hours of incubation. The variation of Optical Density 600 nm (OD_{600}) and the concentration of viable cells varied in parallel during exponential phase and in early stationary phase. However, there was some decoupling between optical density and the concentration of viable cells in later stationary phase. The correlation between OD_{600} and content of viable cells for the initial ten hours was calculated, resulting in a $R^2 = 0.9446$.

Once the intended was that the bacteria was found in the stationary phase, and based on growth kinetics represented below, the cultures were left to growth 12 hours until being submitted to HPP.

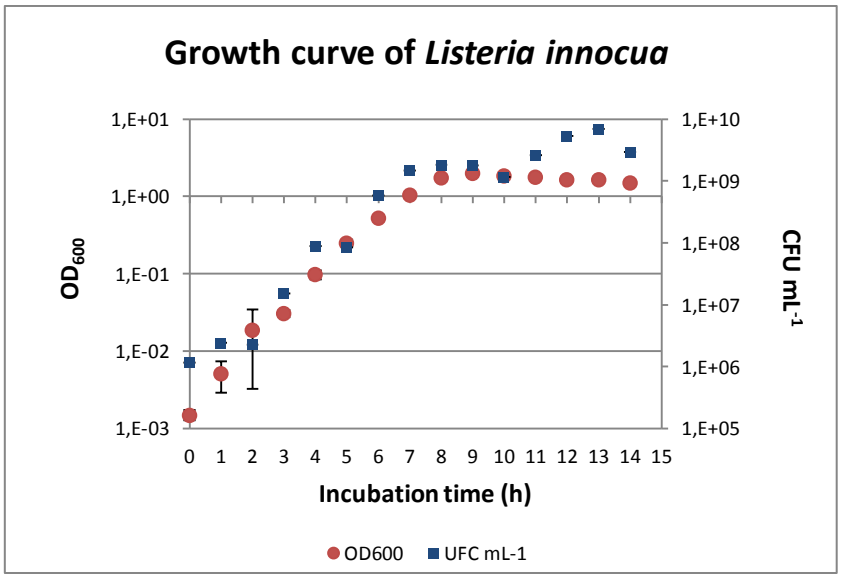

Figure 6 - Graphic representation of OD_{600} and concentration of viable cells during 14 hours of incubation of *Listeria innocua* in TSB at 37 °C with continuous agitation at 170 rpm. Error bars represent the standard deviation.

3.2 High pressure processing (HPP)

3.2.1 Characterization of inactivation kinetics at different pressure values

The preliminary tests of HPP were performed in order to characterize the kinetics of inactivation with different pressure values. Pressures between 100 and 600 MPa were tested with a constant holding time of 5 minutes, and pressurization at room temperature. The values of the inactivation factor (IF) of *L. innocua* corresponding to pressures of 100 MPa, 200 MPa, 300 MPa, 400 MPa, 500 MPa and 600 MPa are represented in figure 7. As expected, the IF increased with the value of pressure. Inactivation to the detection limit (IF > 9 log) was achieved with 400 MPa. The inactivation factors calculated for 100 and 200 MPa were the lowest (0.26 ± 0.29 log and 0.57 ± 0.48 log, respectively) and were not significantly different (P>0.05). At 300 MPa, the IF was 4.36 ± 0.07 log. ANOVA analysis confirmed significant differences between the IF calculated for treatments with 200, 300 and 400 MPa (P<0.05). Multiple comparisons show significant differences between all IF obtained with these three pressure values (Tukey test).

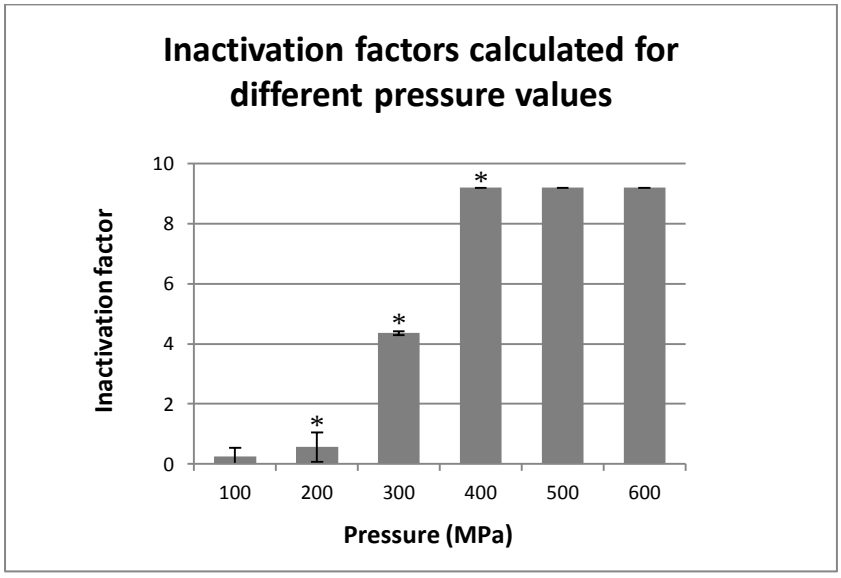

Figure 7 – Inactivation factors calculated for the preliminary tests of inactivation of *Listeria innocua* by HPP at pressure values of 100, 200, 300, 400, 500, and 600 MPa. Columns represent the average inactivation factor calculated for three independent assays and error bars represent the standard deviation. * indicates statistically significant differences (P<0.05).

For the subsequent inactivation experiments, the pressure of 300 MPa was selected as representing the strongest of sublethal effect, and the pressure of 400 MPa was used as the minimum pressure to obtain complete inactivation of *L. innocua*.

3.2.2 Effect of pressurization and depressurization rates and holding time

With the aim of evaluating the influence of the rates of pressurization and depressurization on the inactivation of *L. innocua*, three different pressurization rates were tested, in combination with 1 and 5 minutes of holding time. The values of the pressurization and depressurization rates calculated for each experimental condition are presented in table II. There were small differences between experiments but overall, the low pressurization rate was 1.5-1.6 MPa s^{-1}, the medium pressurization rate was 6.4-6.9 MPa s^{-1} and the high pressurization rate was 10.7-11.9 MPa s^{-1}. The variation of pressure during the assays at 300 and 400 MPa is represented in figure 8.

Table II – Conditions tested for *Listeria innocua* inactivation by HPP.

	Kinetic parameters (MPa s^{-1})	
Pressure values (MPa)	Pressurization rate	Depressurization rate
300	1.5	3.2
	6.4	12.9
	10.7	21.0
400	1.6	3.8
	6.9	14.8
	11.9	23.8

The IF values calculated for treatments with 300 MPa are represented in Figure 9. At 300 MPa, the IF for 1 minute of holding time were < 1 and was not significantly different between the different pressurization/depressurization rates (ANOVA, $P>0.05$). However, for 5 minutes of holding time, there were statistically significant differences (ANOVA, $P<0.05$) in the values of IF corresponding to different pressurization rates. Post-

hoc test allows infer that IF obtained with a low pressurization/depressurization rates is significantly different from those obtained with the other two rates tested. However, there are no differences in inactivation between an intermediate and a high pressurization/depressurization rates. The maximum, although not complete, inactivation, was obtained with low pressurization/depressurization rates (3.17 ± 0.49 log).

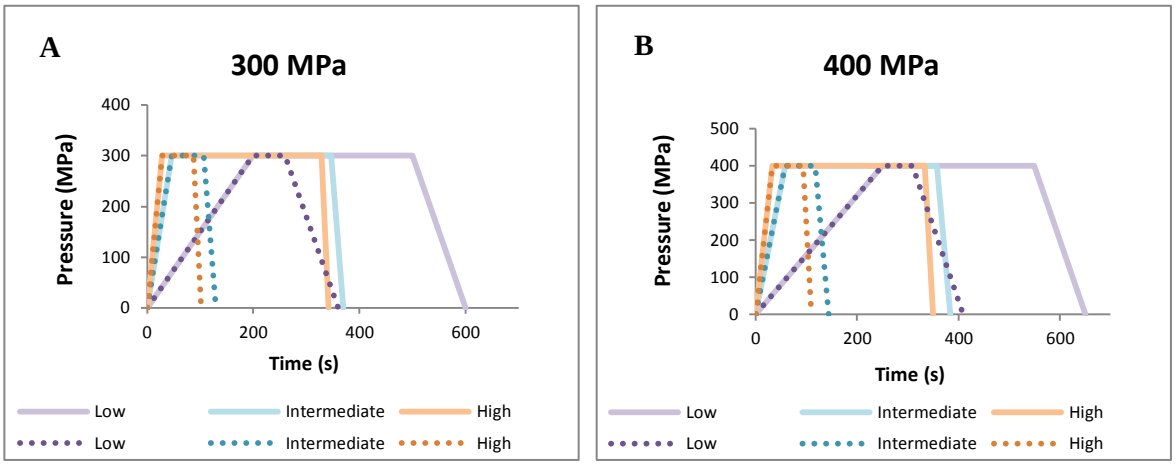

Figure 8 - Pressurization and depressurization profiles at 300 MPa (A) and 400 MPa (B) obtained in assays of inactivation of *Listeria innocua* by HPP. Solid lines represent tests with a holding time of 5 minutes, and dashed lines represent tests with a holding time of 1 minute.

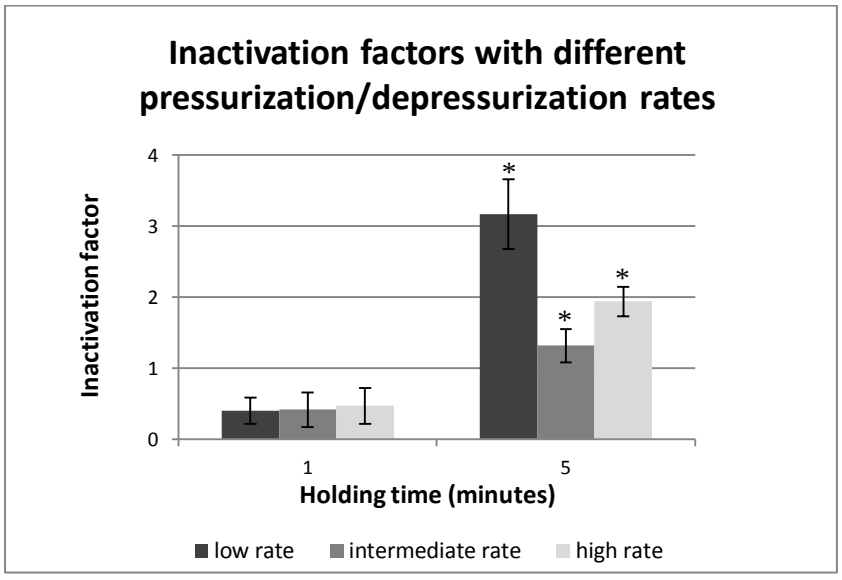

Figure 9 - Inactivation factors obtained in tests of inactivation of *Listeria innocua* by HPP with 300 MPa. Columns represent the average of results obtained in three independent treatments, with three replicate for each pressurization rate tested, and error bars represent the standard deviation. * indicates statistically significant differences (P<0.05)

The IF factors calculated for treatments with 400 MPa are represented in Figure 10. Contrary to what occurred at 300 MPa pressurization/depressurization rates did not have a significant effect (ANOVA, $P>0.05$) on the IF of *L. innocua* at 400 MPa, with 1 or 5 minutes holding time. The maximum IF value obtained was 8.26 ± 1.17 log with high pressurization/depressurization rates and 5 minutes of holding time.

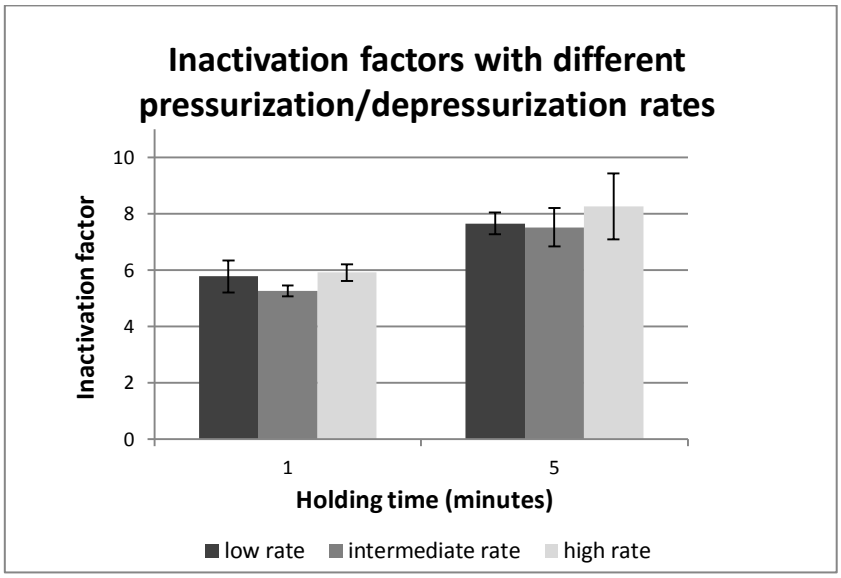

Figure 10 - Inactivation factors obtained in tests of inactivation of *Listeria innocua* by HPP with 400 MPa. Columns represent the average of results obtained in three independent treatments, with three replicate for each pressurization rate tested, and error bars represent the standard deviation.

3.2.3 Recovery of viability after HPP

HPP can be used like a non-thermal food processing technique, for that reason it is important evaluate if *L. innocua* can recover after pressurization, during storage. Recovery tests were performed after submitting bacteria to 400 MPa, with high pressurization/depressurization rates and a holding time of 5 minutes. Treated samples, were stored at 4 °C or 25 °C during 1, 5 and 10 days. The calculated inactivation factors are presented in figure 11. As expected, storage temperature significantly affected the recovery of viability of *L. innocua* after sublethal HPP treatment. Storage at 25 °C caused an almost complete recovery of the culture (Kruskal-Wallis, $P<0.05$). Immediately after treatment, the IF was calculated as 6.91 ± 0.14 log, but after 10 days of incubation at 25 °C

the IF was reduced to 0.28 ± 0.38 log. During storage at 4 °C for 10 days, there was not significant re-growth. The initial IF of 6.91 ± 0.14 log, calculated immediately after treatment was slightly increased to 7.16 ± 0.32 log after 10 days of storage in the cold, a difference that is not statistically significant (ANOVA, $P>0.05$).

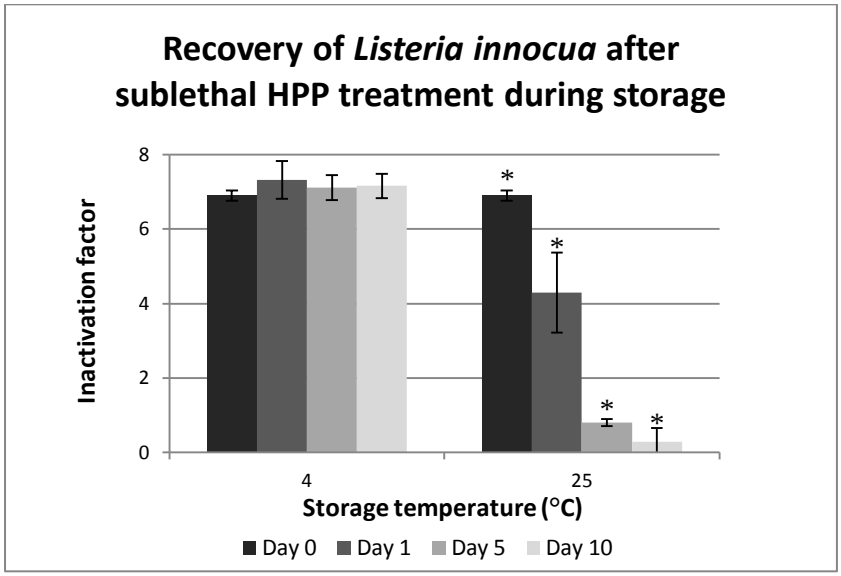

Figure 11 - Inactivation factors calculated for the recovery tests of *Listeria innocua* after HPP at pressure value of 400 MPa, with a high pressurization rate and a holding time of 5 minutes. Columns represent the average of results obtained in three independent assays for each storage day tested, and the error bars represent the standard deviation. * indicates statistically significant differences ($P<0.05$)

In addition, recovery after complete inactivation at 500 MPa was also tested. In this case, storage was only conducted at 25 °C. The inactivation factor calculated immediately after pressurization was 8.30 ± 0.95 log. However, the surviving cells that could not be detected immediately after the treatment grew during the incubation at 25 °C. In this culture, the IF calculated after 10 days of storage (1.05 ± 0.14 log) corresponded to an almost complete recovery. Similarly to what happened after the treatment with 400 MPa, the recovery was detected after the first day and IF values are significantly different (Kruskal-Wallis, $P<0.05$) during the recovery period.

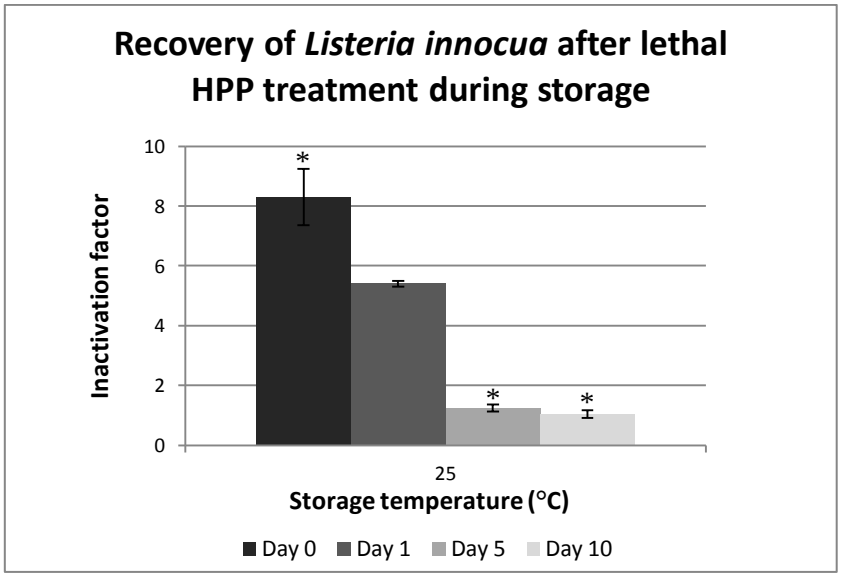

Figure 12 - Inactivation factors calculated for recovery tests of *Listeria innocua* after HPP at pressure value of 500 MPa, with a high pressurization rate and a holding time of 5 minutes. Columns represent the average of results obtained in three independent treatments, with three replicate for each storage day tested, and error bars represent the standard deviation. * indicates statistically significant differences (P<0.05)

4. Discussion

4.1 Characterization of growth kinetics in liquid medium

Bacteria present a characteristic growth curve in which four distinct phases can be distinguished: lag phase, exponential phase, stationary phase and death phase. In this work, because of the conditions created to initiate growth very quickly, lag phase was almost indistinguishable. Cultures inoculated in new medium were quite fresh (≈ 20 hours) without being submitted to long storage periods, being therefore very viable and ready to initiate growth (Hogg, 2005)**.** In addition, the composition of the culture medium was constant, and the new medium was pre-heated to 37 ºC before inoculation, so that temperature changes would not occur. Consequently, cells entered in exponential phase almost immediately.

Bacterial cells are more resistant to stress factors in stationary phase comparatively with exponential phase. For this work, stationary phase *Listeria innocua* cells were used in order to represent a state of greater resistance to pressure (Doona & Feeherry, 2007, Zhang, *et al.*, 2011). From the observation of the growth curve, the beginning of stationary phase was estimated to occur approximately after 9 hours of incubation. Cultures to be used in HPP assays were incubated for 12 hours to ensure full stationary phase. The decoupling between OD_{600} and the concentration of viable cells observed in later stationary phase may be due to some changes in the cellular morphology with aging. Logarithmic death phase was also not observed because incubation was interrupted after 14 hours and at this moment, cultures were still in exponential phase.

4.2 Inactivation of *Listeria innocua* by HPP

HPP has been shown to be an effective method for inactivation of vegetative cells at several temperatures. In this work, pressurization for inactivation of *L. innocua* was conducted at room temperature envisaging the application of this approach as a non-thermal preservation method. Similarly, it represents a "worst-case-scenario" because bacterial cells are more resistant to pressure when applied at temperatures between 20 °C and 30 °C (Alpas, *et al.*, 2000, Raso & Barbosa-Cánovas, 2003, Buzrul, *et al.*, 2008).

4.2.1 Pressure

The efficiency of HPP is determined by the pressure applied, as well as by the holding time to which microorganisms are subjected. In general, the efficiency of bacterial inactivation increases with pressure and holding time (Yuste, *et al.*, 1999, Rendueles, *et al.*, 2011). In the preliminary tests conducted, *L. innocua* was treated at room temperature with different pressure values and a fixed holding time of 5 minutes. As expected, the inactivation efficiency expresses as the IF, increased with increasing pressure until a plateau of complete inactivation was reached.

The inactivation of vegetative forms of bacteria can reach values above 4 log when treatments are carried out at pressures between 400 and 600 MPa at room temperature (Devlieghere, *et al.*, 2004). However, large differences in inactivation levels of foodborne vegetative pathogens are observed, namely between *S. aureus*, *L. monocytogenes*, *E. coli*, *S. enteritidis* and *S. typhimurium* (Alpas, *et al.*, 2000).

The levels of inactivation observed in tests performed in this work is in accordance with the values obtained for *L. innocua* (Alpas, *et al.*, 1998, Buzrul, *et al.*, 2008, Gudbjornsdottir, *et al.*, 2010, Evrendilek & Balasubramaniam, 2011). The differences observed in several works with this species may be attributed to difference in the conditions of pressurization, and in the matrix in which microorganisms are inoculated. The range of inactivation for *L. innocua* varies between from 1-1.5 log in smoked salmon treated with 400/500 MPa (Gudbjornsdottir, *et al.*, 2010), and 2 to 6 log in TSB treated with 325 to 400 MPa (Saucedo-Reyes, *et al.*, 2009).

From the results obtained in this work, 300 MPa was initially chosen as representative of the conditions for sublethal inactivation, and therefore, more convenient to experimentally assess the effect of other parameters on the final outcome of the HPP protocol, expressed in terms of the calculated inactivation factor. However, during the sequence of inactivation experiments, there was a decrease of the IF obtained at 300 MPa, with 5 minutes of holding time and medium pressurization/depressurization rates, with an IF from the preliminary tests of 4.36 ± 0.07 log and from the subsequent tests of 1.32 ± 0.24 log. This may indicate a decreased in the sensitivity of the strain of *L. innocua* to pressure that could be due the repeated cycles of cultivation-conservation in the cold of the inocula used to produce the fresh cultures before each pressurization assay. Some cold-

adaptation might have occurred, and the cold adapted cells selected might also have some enhanced resistance to pressure. For that reason, subsequent tests with different pressurization rates were also carried out at 400 MPa, which in the preliminary tests has caused complete inactivation of the culture.

4.2.2 Pressurization rate and holding time

In addition to pressure and holding time, the efficiency of inactivation by HPP can be influenced by the pressurization and/or depressurization rates used. In this work, different pressurization rates – low, medium and high – were tested. Because of limitations of the equipment, depressurization rates were manipulated in the same way and therefore, a fast pressurization was accompanied by fast depressurization and when pressurization was slow, depressurization was also slow. In addition, 1 and 5 minutes of holding time were also tested, allowing the evaluation of the pressurization/depressurization rates and holding time on the efficiency of *Listeria innocua* inactivation at 300 MPa and 400 MPa.

IF calculated to 300 MPa treatments, for 1 minute, show that there were no significant differences in inactivation between the different rates of pressurization/depressurization. However, when holding time was extended to 5 minutes, there were significant differences in the calculated IF indicating that slow pressurization and depressurization increase the inactivation efficiency in relation to medium and high pressurization/depressurization rates, for equivalent pressure and holding time. These results apparently contradict some results that indicate that slow pressurization leads to lower inactivation, since microbial cells can activate stress response mechanisms (Smelt, 1998). Also, it has been proposed the inactivation efficiency is more affected by depressurization rate than by pressurization rate, and that efficiency of inactivation increases if high depressurization rate are used (Noma, *et al.*, 2002, Chapleau, *et al.*, 2006). However, it is not possible establish a direct comparison, because these works were made with different bacterial species and at higher pressures once that have the aim of obtain maximum inactivation. On the other hand, this work was made with the purpose of evaluate the influence of pressurization/depressurization rates, so lower pressure was applied.

As previously mentioned, IF values obtained with the medium pressurization rate (the same used in the first set of preliminary tests) with 5 minutes of holding time were lower than those obtained in preliminary tests indicating that the batch culture may have developed some resistance to pressure during repeated storage at 4 °C. The same happened in the assays with 400 MPa, in which the expected complete inactivation did not occur.

IF calculated for treatments with 400 MPa did not indicate significant differences between different pressurization/depressurization rates, for holding times of 1 or 5 minutes, contrary to what was observed for treatments with 300 MPa. Although the difference was not significant, the highest IF corresponded to fast pressurization/depressurization.

The holding time has a significant effect, on the inactivation efficiency, particularly in the treatments with 300 MPa. The inactivation factor with 5 minutes was 8 times higher than with 1 minute for slow pressurization/depressurization, 3 times for intermediate pressurization/depressurization, and approximately 4 times higher for high rates of pressurization and depressurization. However, the increase in pressure seems to have a strongest effect on the inactivation efficiency than the increase in holding time. Still, there is slightly differences in the rates of pressurization/depressurization at 300 Ma and 400 MPa that do not allow check if 1 minute at 400 MPa caused a strongest inactivating effect than 5 minutes at 300 MPa.

4.2.3 Recovery test after HPP

HPP is a method that can be applied to food processing for the inactivation of pathogenic microorganisms. However, in addition to the assessment of the inactivation efficiency of a particular HPP in terms of the reduction of the concentration of viable cells in the sample, it is also important to understand if recovery from sublethal damage in treated cells will occur during storage in refrigerated or room temperature conditions, or even if, on the contrary, damaged cells will ultimately be completely inactivated in the post-treatment period.

L. innocua, used in this work, is a surrogate of *L. monocytogenes*, a foodborne pathogen, because it presents similar features, so can also be used to evaluate the possible recovery of *L. monocytogenes* after processing of food. For these assays, the experimental

HPP conditions selected were those causing the highest sublethal inactivation: 400 MPa during 5 minutes with high pressurization/depressurization rates. IF was calculated at day 0 (6.91 ± 0.14 log) immediately after treatment, and treated samples were stored , at 4 ºC or at 25 ºC during 1, 5 and 10 days.

During storage at 4 ºC there the IF values remained fairly constant indicating that the treated culture failed to recover and reinitiate growth. The results of this work confirm previous information that *L. innocua* and *L. monocytogenes* cells remain inactive during long chilling times, failing to recover at this storage temperature after high pressure treatment (Yuste, *et al.*, 1998, Yuste, *et al.*, 1999, Bull, *et al.*, 2005).

On the other hand, during storage at 25 ºC there was an almost complete recovery indicating that some damaged bacteria may have recovered or that some scarce cells persisted although their concentration was below the detection limit of the culture-dependent approach followed. After 10 days at 25 ºC, the concentration of viable cells in the treated cultures was very similar to the initial concentration and IF values were close to zero (0.28 ± 0.58 log).

For these assays also selected the experimental HPP conditions that cause lethal inactivation: 500 MPa during 5 minutes with high pressurization/depressurization rates. After the development of some rare persistent cells is confirmed by the results obtained in samples treated with 500 MPa, which caused inactivation to the detection limit (8.30 ± 0.95 log). After recovery at 25 ºC the inactivation factor almost observed for null such has as observed in the cell suspensions treated with 400 MPa.

The fact that there is recovery after HPP when samples are stored at 25 ºC suggests that *L. innocua* may be sublethally injured when submitted to high pressure, as proposed for *L. monocytogenes* in other studies (Ritz, *et al.*, 2002, Bull, *et al.*, 2005). Data suggest that the recovery may happen in two phases. Initially the membrane is repaired and, in a second phase there is a physiological damage repair (intracellular components). This theory rejects the hypothesis that cells are able to repair the damage done by HPP when stored at 4 ºC, which is in agreement with the results data obtained in this work. The second phase could only happen at incubation temperatures above 15 ºC, with a complete repair of physiological damage, allowing bacterial to grow rapidly (Bull, *et al.*, 2005). This theory, despite lack of proven research and data, seems to be a valid explanation for what has been observed in these recovery tests, at 400 and 500 MPa.

5. Conclusion

From the results of this work, it is possible to conclude that depending on the pressure applied and on the holding time, the pressurization/depressurization rates may or may not be a relevant parameter in determining the inactivation efficiency.

The application of a low pressurization rate followed by high pressure at 300 MPa during 5 minutes, and a low depressurization rate, lead to enhanced inactivation of *Listeria innocua* in comparison with the other condition tested at this relatively low pressure. At 400 MPa, that initially inactivated the test strain to the detection limit, the pressurization/depressurization rates did not have a significant effect on the final inactivation factor.

Increasing holding time also increase the inactivation efficiency. However, for the rather short holding times tested in this study (1 and 5 minutes), pressure seems to have a greater relative effect than holding time.

The assessment of the eventual recovery of viability of treated cells during storage indicated that if stored at refrigerator temperatures *L .innocua* does not recover and cannot grow, and therefore the concentration of viable cells remains stable. However, if stored at 25 °C, there is a complete recovery independently of the HPP (400 or 500 MPa). This implies that even in the cases in which inactivation of the pathogen to the detection limit is achieved with a suitable HPP protocol, the condition in which the product is stored after treatment are of the outmost importance in the extension of shelf life and in the preservation of food quality.

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