Variability of *Listeria monocytogenes* response to high hydrostatic pressure.

A case study on meat products

PhD Thesis

Diego Wilches Pérez

Burgos, 2016

UNIVERSIDAD DE BURGOS

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Memoria presentada por

Diego Wilches Pérez

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DEPARTAMENTO DE BIOTECNOLOGÍA Y CIENCIA DE LOS ALIMENTOS

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INFORMAN:

Favorablemente la presentación de dicha tesis, ya que reúne las condiciones necesarias para su defensa en cuanto a la realización de la fase experimental y la elaboración de la memoria.

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En Burgos, a 3 de Noviembre de 2015

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Que la memoria titulada **"Variability of *Listeria monocytogenes* response to high hydrostatic pressure. A case study on meat products / Variabilidad de la respuesta de *Listeria monocytogenes* a las altas presiones hidrostáticas. Un caso de estudio en productos cárnicos"**presentada por **Don Diego Fernando Wilches Pérez**, ha sido realizada en la en el Área de Tecnología de los Alimentos bajo la dirección del Doctor Jordi Rovira Carballido, Profesor titular del Área, la Doctora Isabel Jaime Moreno, Profesor titular del Área, y la Doctora Carole Tonello, Directora del Departamento de Aplicaciones y Procesos Alimentarios de Hiperbaric S.A. y en representación de la Comisión del Título, autoriza su presentación para ser defendida como Tesis Doctoral.

Y para que conste, y a efectos oportunos, firmo el presente certificado,

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Esta tesis se ha realizado gracias a la concesión de la ayuda Caja de Burgos para la Formación de Personal Investigador de la Universidad de Burgos

A mi familia

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Summary

According to World Health Organization, unsafe food contaminated with harmful bacteria, viruses, parasites or chemical substances, causes more than 200 diseases. Annually, around 2 million people died by the consequences of food- and waterborne diseases. Foodborne diseases are a threat for the socioeconomic development of the regions, straining health care systems, leading to malnutrition particularly in infants, children, elderly and immune-compromised people. Globalization has triggered growing consumer demand for a wider variety of foods, resulting in an increasingly complex food chain. In addition, changes in consumer habits have increased the number of people consuming ready-to-eat food products, increasing the risk of contaminated food with *Listeria monocytogenes*.

Since 1980s, after the first food-related outbreaks, the control of the pathogen has become a priority for food safety authorities worldwide because of its consequences on human health. Listeriosis has one of the highest rates of hospitalization and case-fatality among foodborne diseases.

One of the technologies for controlling *L. monocytogenes* is high pressure processing. The lethal effect of this non-thermal technology on microorganisms is due to the molecular changes induced by high hydrostatic pressure up to 600 MPa. The technology has demonstrated to be efficient in the control of *Listeria monocytogenes*. However, the efficacy of the technology as a preservation method depends on the intra-species diversity of the target pathogen and the food matrix. While strain variability gives to *Listeria monocytogenes* the ability to withstand different stress conditions, food matrix and its components can exert a baroprotective effect. This makes challenging the selection of processing conditions that allow a considerable inactivation of the pathogen. Therefore, we hypothesized that the response of *Listeria monocytogenes* to high pressure processing depends on the strain, and this variability among strains could be influenced by both the food matrix and the processing conditions.

The first part of the work consisted of the selection of a representative set of *Listeria monocytogenes* strains for covering the natural diversity of the pathogen. Thirty out of the initial 120 strains from three *L. monocytogenes* collections were selected based on the geno- and phenotypical characterization done and the information provided by collections' hosts. The thirty strains represented the four lineages, the four serogroups, human-clinical and foodborne, epidemic and sporadic-related, and diverse virulence-factors of *Listeria monocytogenes.*

Following, the strain characterization and selection, the response (inactivation and sublethal) of these strains to high hydrostatic pressure in a model system was evaluated. The pressure resistance of *L. monocytogenes* in a buffer varied among strains depending on the high pressure processing conditions. The strain variability was higher at intermediate processing conditions. Most of the strains exhibited a low-intermediate resistance, existing three strains considerably more pressure-tolerant than the rest. Pressure-resistance of the strains did not correlate with geno- and phenotypical characteristics (lineage, serogroup, virulence factors, pathogenicity or origin).

Since food matrices influence the lethal effect of high pressure processing, the strain variability of *Listeria monocytogenes* to high pressure was evaluated but now, in meat matrices. Cooked ham and dry-cured ham were selected based on its importance in Spanish and European meat industry and the problems associated to the presence of *Listeria monocytogenes* in these products. The pressure-resistance of 15 meat-borne and human clinic strains was evaluated. The pathogen exhibited differences in its pressure-tolerance at strain level. The strain variability depended on the holding time at 600 MPa and the meat matrix, finding a larger number of pressure-resistant strains in dry-cured ham than in cooked ham. These findings could indicate a cross-protection phenomenon between salt and hydrostatic pressure stresses. Pressure-resistance of the pathogen strains, in both meat products, showed no correlation with lineage, serogroup, virulence factors, pathogenicity or origin.

The results of this PhD thesis about strain variability of the pathogen allowed the design of a cocktail comprised of five pressure-resistant strains of *L. monocytogenes* for being used in challenge tests of high pressure-processed ready-to-eat (RTE) meat products.

The cocktail was resistant to high hydrostatic pressure in most of the evaluated meat matrices except in dry-fermented products, probably due to an acid sensitivity of one or more of the strains included.

A meatborne strain of *Listeria innocua* was included in strain variability evaluation for comparison purposes. *L. innocua* UBU strain exhibited a high pressure-resistance comparable to that exhibited by the most pressure-tolerant *L. monocytogenes* strains in the buffer system as well as in cooked and dry-cured ham. The inactivation levels of *Listeria innocua* UBU were similar to or lower than the levels of the proposed cocktail of five-strain pressure-resistant *L. monocytogenes* in raw, cooked, cured and dry-fermented meat products. This results suggested that this strain of *L. innocua* can be used as a surrogate of *L. monocytogenes* in diverse high pressure-processed meat products.

In conclusion, the response of *Listeria monocytogenes* to high pressure processing differs strain-to-strain. This variability is influenced by the characteristics of the food matrix and the processing conditions, pressure and holding time. The knowledge in strain variability allows the design of appropriate cocktails and the selection of suitable surrogate microorganisms to use in validation studies of high pressure-processed meat products.

Resumen

Según la Organización Mundial de la Salud, los alimentos contaminados con bacterias, virus, parásitos o sustancias químicas, son la causa de más de 200 enfermedades. Se estima que aproximadamente dos millones de personas mueren anualmente como consecuencia de enfermedades relacionadas con el consumo de agua o los alimentos contaminados. Las enfermedades alimentarias son una amenaza para el desarrollo socioeconómico de las regiones, en tanto que desgastan los sistemas de atención sanitaria y conducen a malnutrición, fundamentalmente entre los grupos de población de niños, mayores e inmunodeprimidos. La globalización ha desencadenado una creciente demanda en la variedad alimentaria lo que resulta en una cadena alimentaria cada vez más compleja. Además, los cambios en el estilo de vida de la población, han incrementado el consumo de productos listos para consumir, lo que genera un aumento del riesgo de contaminación alimentaria por *Listeria monocytogenes*.

Desde 1980s, tras los primeros brotes alimentarios, el control de dicho patógeno se ha convertido en una prioridad para las autoridades de seguridad alimentaria a nivel mundial, por las graves consecuencias que ocasiona sobre la salud humana. Dentro de las enfermedades alimentarias, la listeriosis genera una de las mayores tasas de hospitalización y mortalidad.

Una de las tecnologías usadas para el control de *L. monocytogenes* es el procesamiento por altas presiones. El efecto letal sobre los microorganismos que ocasiona dicha tecnología se debe a los cambios moleculares inducidos por altas presiones hidrostáticas (hasta 600 MPa). Esta tecnología no térmica ha demostrado ser eficaz en el control de *Listeria monocytogenes*. Sin embargo, tal eficacia depende de la diversidad intra-especie del patógeno objetivo y de la matriz alimentaria. Variaciones a nivel de cepa son las responsables de la habilidad que caracteriza a *Listeria monocytogenes* para soportar distintas condiciones de estrés. La matriz alimentaria y los componentes de la misma pueden ejercer un efecto baroprotector. Por todo ello, la selección de aquellas

condiciones de procesamiento que permitan una considerable inactivación del patógeno constituye un gran desafío para la industria. Por tanto, en la presente Tesis Doctoral se hipotetiza que la respuesta de *Listeria monocytogenes* al procesamiento por altas presiones depende de la cepa y que dicha variabilidad cepa a cepa podría estar afectada por la matriz alimentaria y las condiciones de procesamiento.

La primera parte del trabajo consistió en la selección de un número de cepas representativo de *L. monocytogenes* para cubrir la diversidad natural del patógeno. De las 120 cepas de partida pertenecientes a tres colecciones distintas, se escogieron 30 cepas. La selección se basó en una caracterización geno- y fenotípica y en la información en parte proporcionada por los proveedores de las colecciones. Las 30 cepas son representativas de los 4 linajes, de los 4 serogrupos, aisladas de casos clínicos y de alimentos, de brotes epidémicos y casos esporádicos y de diversos factores de virulencia de *Listeria monocytogenes.*

Tras la caracterización y selección de las cepas, se evaluó la respuesta (inactivación y daño subletal) de las cepas a altas presiones hidrostáticas en un buffer. La resistencia de *L. monocytogenes* en buffer se mostró variable cepa a cepa, dependiendo de las condiciones de procesamiento. La variabilidad entre cepas fue más notable en las condiciones intermedias de procesamiento. La mayoría de las cepas ofreció una resistencia intermedia-baja, observándose tres cepas considerablemente más tolerantes a la presión que el resto. La resistencia a la presión de las cepas no correlacionó con sus características geno- y fenotípicas (linaje, serogrupo, factores de virulencia, patogenicidad u origen).

Teniendo en cuenta que las matrices alimentarias influyen en el efecto letal generado por el procesamiento basado en altas presiones, se determinó la variabilidad cepa a cepa de la respuesta de *Listeria monocytogenes* frente a altas presiones, en este caso en matrices cárnicas. Se seleccionaron jamón cocido y jamón curado dada la importancia de estos productos en la industria cárnica española y los problemas asociados con la presencia de *L. monocytogenes* en los mismos. Se determinó la resistencia a la presión de 15 cepas de origen clínico y cárnico, observándose diferencias en la tolerancia a la presión a nivel de cepa. La variabilidad entre cepas dependió del tiempo de

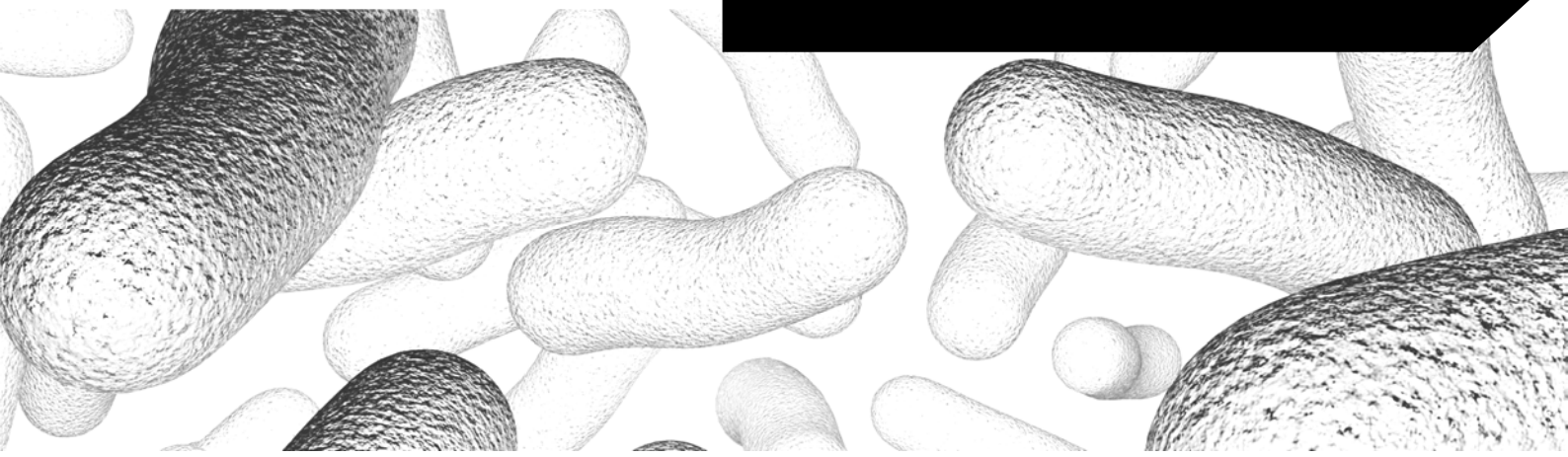
procesamiento (a 600 MPa) y de la matriz cárnica, encontrándose un mayor número de cepas resistentes a la presión en el jamón curado que en el cocido. Esto podría indicar un fenómeno de protección cruzada entre el estrés osmótico y el causado por la presión hidrostática. No se encontraron correlaciones entre la resistencia a la presión de las cepas de *L. monocytogenes* y características como el linaje, serogrupo, factores de virulencia, patogenicidad u orígen, para ninguno de los productos cárnicos estudiados.

Los resultados de esta Tesis Doctoral sobre la variabilidad cepa a cepa del patógeno en cuestión, permitieron el diseño de un cóctel formado por cinco cepas resistentes a la presión, con el objeto de ser empleado en *challenge tests* de productos cárnicos procesados por altas presiones. El cóctel propuesto demostró ser resistente frente a altas presiones hidrostáticas en la mayoría de las matrices cárnicas evaluadas, con la excepción de los cárnicos curados-fermentados, probablemente debido a la sensibilidad a la acidez de una o más de las cepas incluidas.

Una cepa de *Listeria innocua* aislada de carne fue incluida a lo largo del estudio con fines comparativos. La cepa denominada *L. innocua* UBU mostró una resistencia a la alta presión comparable con la correspondiente a las cepas de *L. monocytogenes* más tolerantes, tanto en buffer como en jamón cocido y curado. Los niveles de inactivación de *L. innocua* UBU fueron similares o menores que los del cóctel propuesto, en productos cárnicos crudos, cocidos, curados y curados-fermentados. Estos resultados sugieren que la cepa de *L. innocua* estudiada puede ser usada como *surrogate* de *Listeria monocytogenes* en diversos productos cárnicos procesados por altas presiones.

En conclusión, la respuesta de *Listeria monocytogenes* frente a altas presiones difiere entre cepas. Dicha variabilidad se ve afectada por las características de la matriz alimentaria y las condiciones de procesamiento, presión y tiempo. El conocimiento relativo a la variabilidad cepa a cepa permite el diseño apropiado de cócteles y la selección de *surrogates* adecuados para ser empleados en estudios de validación de productos cárnicos procesados por altas presiones.

Literature review



Literature review

The importance of *L. monocytogenes* in the food industry

Overview of *L. monocytogenes* and listeriosis

Hülpers, in 1911, identified a Gram-positive bacteria from rabbit liver necrosis and was named *Bacillus hepatica.* Fifteen years later, Murray, Webb and Swann isolated a bacterium from dead laboratory rabbits, which caused monocytosis in leukocytes. They called *Bacterium monocytogenes* to the pathogen. One year later, an isolate from wild gerbils induced the same liver infections in experimentally infected animals, and denominated *Listerella hepatolytica* to honor Lord Joseph Lister. Since 1940, the bacterium name *Listeria monocytogenes* was established for taxonomical reasons (Pirie, 1940).

L. monocytogenes is a Gram-positive, facultative anaerobic, catalase positive rod shaped bacterium. The microorganism belongs to *Listeria* genus, which is composed of other nine species: *L. innocua, L. seeligeri, L. rocourtiae, L. marthii, L. welshimeri, L. fleischmannii, L. weihenstephanensis, L. grayi* and *L. ivanovii* (Bourdichon, 2014). Generally, the only species considered pathogenic to humans is *L. monocytogenes*. However, some reports stated that *L. grayi* and *L. ivanovii* can cause disease in humans (Guillet et al., 2010; Todeschini et al., 1998).

In 1929, Nyfelt reported the first confirmed isolation from humans. However, *Listeria monocytogenes* is considered to be a major foodborne pathogen since the coleslaw outbreak in 1981 (Bourdichon, 2014). Prior to the 1980's, listeriosis, the disease in humans caused by this pathogen was a rarity, as it was considered an animal pathogen with a few of human cases (Abu Mraheil, 2013; Warriner and Namvar, 2009). The pathogen causes serious localized and generalized infections depending on the group. For healthy adults, listeriosis is mainly manifested as diarrhea and fever. The disease is severe for pregnant women and newborns. For the former, symptoms include fever,

diarrhea, abortion, or stillbirth; and septicemia, pneumonia, or meningitis are manifested in newborns (Todd and Notermans, 2011). Persons with a predisposing condition having an immunocompromised status are especially susceptible to the disease. People with transplant, cancer or AIDS are hundreds times more susceptible to acquire listeriosis than healthy individuals. Even, the risk of the disease is higher in people with diabetes (30 times), alcoholism (18 times) and over 65 years old (7.5 times) than healthy population according to French epidemiological data (FAO, 2004).

After the consumption of contaminated foods, infection involves, first, the survival through the gastric passage, followed by the survival and colonization in the intestinal tract, and the invasion of intestinal epithelial cells. The infection steps are modulated by several regulatory mechanisms controlled by virulence genes that contribute to its ability to withstand with changing environmental conditions (Chaturongakul et al., 2008). The same biological mechanisms allow it to be worldwide distributed with a high prevalence in distinct world regions (Chenal-Francisque et al., 2011) and ubiquitously distributed throughout the environment including soil, water, sewage, vegetation, and wild animal feces as well as on the farm and in food-processing facilities (Sauders et al., 2012).

The meta-analysis done by de Noordhout et al. (2014) estimated that the pathogen caused 23,150 illness and 5,463 deaths worldwide in 2010. The highest estimated listeriosis incidence was Latin America region (0.469 cases per 100,000 population) and the lowest incidence in East Europe zone (0.042 cases per 100,000 population). Most cases are reported in high-income countries, but it is not clear whether the differences reflect true geographical differences, food habits and handling or differences in diagnosis and reporting practices (FAO, 2004). In those regions, incidence is quite low but fatality rate is high. For instance, 1,763 confirmed human cases (0.44 cases per 100,000 population) were reported in Europe during 2013, which represented an 8.6 % increase compared with 2012. The EU-hospitalization rate (99.1 %) and the case-fatality rate (15.6 %) associated to *L. monocytogenes* was the highest among all zoonosis reported (EFSA, 2015). In United States, the epidemiological data reported similar values to European for the same period. Although incidence of listeriosis was low (0.24 cases

per 100,000 population), the hospitalization rate (91 %) and case-fatality rate (19.5 %) were the highest among foodborne diseases (Crim et al., 2014).

Food as a primary route of transmission of the pathogen

The most of infections (99%) caused by *L. monocytogenes* are thought to be foodborne (Swaminathan and Gerner-Smidt, 2007). The pathogen tolerates harsh conditions, growing at wide range of temperature (-1.5 °C to 45 °C), pH (4.3 to 9.1) and even high salt concentrations, which allows being found in different types of foods (Bourdichon, 2014). Besides, *L. monocytogenes* has the ability to adhere on diverse surfaces found in food-processing plants including stainless steel, rubber, glass, and polypropylene resulting in biofilm formation (Lunden et al., 2000). Its high tolerance and capacity to survive and grow in different food products and environments makes the food products the primary route of human transmission. Foods with high levels of contamination associated with the vast majority of cases of listeriosis, increasing the risk of disease from 100- to 1000-fold on a per serving basis (FAO, 2004).

According to the International Life Sciences Institute Risk Science Institute Expert Panel, high-risk foods for causing listeriosis are characterized by: having the potential for contamination with *L. monocytogenes*, supporting the growth to high numbers, being ready-to-eat (RTE) food, requiring refrigeration; and being stored for an extended period of time (ILSI, 2005). The latter led to establish a microbiological criteria for *L. monocytogenes* in RTE food products, which vary around the world. In the *Codex* member states and European Union, RTE foods in which growth will not occur, rejection level is set at 100 colony-forming units (CFU) per gram, whereas those products in which growth can occur, absence in 25 g is required. In addition, Commission Regulation (EC) No 2073/2005 sets a complementary introduction of criterion (absence in 25 g) for infant products and RTE foods for special medical purposes (Luber, 2011; The Commission of the European Communities, 2005). The policy on *L. monocytogenes* in RTE foods in Canada is aligned on the European approach (Health Canada, 2011),

whereas The United States Department for Agriculture maintains a policy of 'zero-tolerance' for *L. monocytogenes* in RTE foods (USDA-FSIS, 2015).

In regard of meat products, *L. monocytogenes* is found in raw materials, processing lines and in final meat products (Baer et al., 2013), as in small and medium-sized as well as large-sized establishments (Pérez-Rodríguez et al., 2010). The role of the processing machines in meat processing contamination and in contaminating meat products appeared to be important because the final product of several processing lines was contaminated with the same strains of *L. monocytogenes* that found in the processing machines (Lundén et al., 2003). According to RASFF, the European Rapid Alert System for Food and Feed, since 2010, a mean of 14 alerts about the presence of *Listeria monocytogenes* in RTE meat products are annually notified (RASFF, 2015). The pathogen were detected in less than 4 % of poultry, bovine and porcine meat products samples, in Europe during 2013 (EFSA, 2015). However, the levels above 100 CFU/g were enumerated in less than 1 % of the tested units. During the same year, 12 *Listeria* outbreaks were reported by the EU State members. Only one of them were related to the consumption of contaminated RTE meat products. Since 1992, meat products such as deli meats, sliced cook meats, sausages have been associated to several *Listeria monocytogenes* outbreaks in Europe, North America and Oceania (NSW food authority, 2013).

Economic impact of *L. monocytogenes*

Since the severity of listeriosis, its high hospitalization and cost-fatality rate, the disease entails high health-associated costs. Taking into account direct healthcare, direct non-healthcare and indirect non-healthcare costs, the cost-of-illness of *L. monocytogenes* was the highest among the 18 foodborne pathogens reported in the Netherlands in 2011 (Mangen et al., 2015). The health-associated cost reached € 106,922 per case, being around 23, 141 and 168 times higher than the cost of illness associated to Shiga-toxin *Escherichia coli, Campylobacter* spp. and *Salmonella* spp., respectively. *Listeria monocytogenes* ranked third among 15 pathogens in terms of total economic burden in

USA, estimated in USD 2.8 billion in economic burden in a typical year (Hoffmann et al., 2015). However, the low number of cases made this foodborne pathogen the second costly disease, reaching USD 1.8 million per case.

Although there is no human disease, food contamination with the pathogen can lead to high cost due to product recall. The annual cost of food product recalls due to *L. monocytogenes* are estimated to be between USD 1.2 and 2.4 billion in the USA (Ivanek et al., 2004). In the recent *Listeria* outbreak linked to Blue Bell ice cream, the analysts estimated the shutdown of the production lines has cost more than USD 200 million (Moore, 2015). Besides product recalls, market access can be limited by the presence of the microorganism. On dry-cured meats, typical European highly appreciated foods by consumers, the pathogen is not able to grow on them. However, the presence of the pathogen constitutes an impediment of export to those countries with a zero-tolerance policy such as USA and Japan (Bover-Cid et al., 2011). For instance, dry-cured hams from Spain and Italy failed eight times during 2010 – 2012 at the Australian border due to the presence of the microorganism, not being allowed their entrance to the country (NSW food authority, 2013). Spanish dry-cured ham market for exportation represents € 237.5 million (Cruz, 2013). Thus, public health concerns and the access to markets are the main drive for controlling *L. monocytogenes* in meat industry.

Controlling *L. monocytogenes* in meat industry

Technologies for controlling the pathogen in RTE meat products

The most effective strategies to control *L. monocytogenes* in high-risk foods include good manufacturing practices, sanitation procedures, hazard analysis critical control point programs, intensive environmental sampling program, for minimizing environmental contamination and to prevent cross-contamination (ILSI, 2005). In addition, The ILSI Expert Panel proposed time and temperature controls throughout the entire distribution and storage period, the reformulation to prevent or delay the growth

of the microorganism and using postpackaging treatments to destroy the pathogen on products. Preservation methods such as thermal treatments, non-thermal technologies, antimicrobials and biopreservation have been proposed to control *L. monocytogenes* in RTE food products (Baer et al., 2013; Jiang and Xiong, 2014; Zhu et al., 2005).

Although *L. monocytogenes* is more thermo-tolerant than other pathogens, it is inactivated when heated above 70 °C, making to thermal treatment the most common intervention strategy against the pathogen (Thévenot et al., 2006). As postpackage pasteurization, RTE meats are commonly submerged in hot water or steam pasteurized. Treatments at temperatures above 90 °C for few minutes inactivated up to 4 log cycles of *L. monocytogenes* in deli meats (Muriana et al., 2002). By using steam was possible to achieve 2 log-inactivation in seconds in sausages, without affecting sensory quality of the meat product (Murphy et al., 2005). Despite the considerable listericidal effect, the roughness of the product surface and package size influenced its efficacy (Muriana et al., 2002).

The use of antimicrobials as additives to control pathogens in RTE meats is considered a novel approach to food safety, which can be applied either by direct incorporation as product formulation ingredients or by spray on the product surface before packaging (Jiang and Xiong, 2014). These compounds have the ability to affect cellular metabolism and/or membrane through varied biochemical mechanisms, leading to a bacteriostatic or bactericidal effect depending on the dose and the chemical nature of the antimicrobial (Baer et al., 2013). Organic acids and their salts, plant extracts, lactoferrins and other compounds have demonstrated to be efficient to control the pathogen in RTE meats as Jiang and Xiong (2014) summarized. Their effect on sensory quality and the demands of consumer on “clean-labelled” RTE meat products limit the application of the technology.

As an alternative to chemical preservatives, biopreservation has been suggested to control *L. monocytogenes.* Some strains of lactic acid bacteria control successfully the presence of the pathogen, mainly due to the production of bacteriocins, polypeptides with a wide bacteriostatic and bactericidal activity (Jacobsen et al., 2003). Bacteriocins such as nisin, reuterin, sakacin and enterocin have demonstrated antilisterial activity.

However, the regulation is hampering the application in purified form, only allowing the addition of nisin (Hugas, 2003).

Among non-thermal physical technologies, irradiation has demonstrated effectiveness against meat-borne pathogens in RTE meats, including *L. monocytogenes* (Hoz et al., 2008; Uesugi and Moraru, 2009). Ionizing radiation (γ-ray, X-ray, and electron beam) and UV light mainly target the molecular bonds in the microbial DNA thereby affecting the synthesis of the nucleic acid (Ahn and Lee, 2012). Despite the demonstrated effectiveness against *Listeria* in RTE meats, irradiation has not been approved for commercial applications on this particular food group.

High pressure processing. An overview

FDA (2000) describes High Pressure Processing (HPP) as a food preservation technology, which subjects liquid and solid foods, with or without packaging, to pressures between 100 and 800 MPa, during millisecond pulse to a treatment time of over 1200 s. However, at industrial level, pressure ranges between 400 and 600 MPa for holding times between few seconds and 600 s.

High-pressure technology is mostly related to material science. However, in 1889, Hite successfully pressurized certain beverages and found that spoilage was delayed due to bacterial inactivation. Later, food scientists made preliminary trials to inactivate microorganisms in other food products in the early 1900s but research was limited due to lack of adequate equipment. It was not until the early 1990s that research of this technology was a viable tool for food preservation (Bermúdez-Aguirre and Barbosa-Cánovas, 2011). Jams, jellies and sauces, in Japan, and guacamole, in USA, were the first high pressure-processed products marketed to consumers in the early 1990s. Nowadays, there are about 350 HPP industrial machines running in vegetable, meat, dairy, seafood and juice food industries, with a global production estimated in 600 million kg per year.

The technology is governed by two principles, the isostatic and Le Chatelier's principles (Campus, 2010). Water is commonly used as pressurization fluid in industrial HPP machines, which transmits pressure isostatically to the food product almost instantaneously and uniformly. Therefore, the efficacy of the process does not depend on the product size and geometry because the isostatic pressure transmission is not mass-/time dependent. The latter facilitates the scale up from laboratory findings to commercial applications (Németh et al., 2015). According to Le Chatelier's principle, the application of pressure shifts an equilibrium to the state that occupies the smallest volume. Covalent bonds are not affected by high hydrostatic pressure, due to their low compressibility and do not break within the ranges of pressures normally used in food. Therefore, vitamins, amino acids, flavor molecules, antioxidants and other low-molecular-weight compounds are minimally affected, in consequence, the nutritional and sensory quality of foods are slightly altered (Farkas and Hoover, 2000). While hydrogen bond formation is stabilized by pressure, ionic bonds and hydrophobic interactions are disrupted (Considine et al., 2008). Since the electrostatic and hydrophobic interactions maintain the tertiary structure of proteins, the changes induced by high hydrostatic pressure on these macromolecules determine the effect of pressure on organisms at cellular level (Campus, 2010).

Different cellular systems and structures, dependent or composed by proteins, are susceptible to be affected by high pressure, even at pressures around 10 MPa (**Table 1**, Abe, 2007). As the pressure increases, more systems and structures can be altered, causing damages which can lead to cell death.

The scientific evidence suggests that pressure-induced cell death is a consequence of multiple damages, which can be attributed to four factors: (i) denaturation of proteins and enzymes, (ii) phase transition and change in fluidity of cell membranes, (iii) breaking down of ribosomes in subunits, and (iv) changes in intracellular pH caused by enzyme denaturation and membrane damage (Georget et al., 2015; Huang et al., 2014). However, it remains unclear whether these targets are simultaneously of sequentially affected during high pressure processing (Gänzle and Liu, 2015).

Table 1. Cellular processes and structures affected by high hydrostatic pressure (Abe, 2007)

Cellular process / structure	Inhibitory pressure (MPa <)
Motility	10
Cell division	20
Nutrient uptake	15 - 20
Ethanol fermentation	25 - 50
Membrane protein function	25 – 50
Replication	50
Transcription	50 – 100
Protein synthesis	50
Protein oligomerization	50 – 100
Soluble protein function	100
Viability	100 – 200
Protein structure (monomer)	200
DNA structure (double helix)	1,000

Physiological, morphological and genetic effects of high pressure on *Listeria monocytogenes*

In general, cell membrane is considered the first organelle to be damaged by pressure. When this structure is damaged, nutrients intake and waste elimination is hindered, and normal metabolic pathways are altered (Torres and Velazquez, 2005). The determinations of membrane integrity by flow cytometry demonstrated that membrane potential of *L. monocytogenes* cells decreased after 400 MPa for 10 min (Ritz et al., 2002). This alteration indicates that cell ability to maintain homeostasis is affected, explaining the changes in intracellular pH, potassium and ATP (Tholozan et al., 2000), probably due to the denaturation of the proteins that mediate proton flow and synthesis of ATP (Huang et al., 2014). Although the cell membrane is damaged by high hydrostatic pressure, *L. monocytogenes* cell volume was not significantly affected (Ritz et al., 2006, 2002; Tholozan et al., 2000). However, pressure can inflict the visible damage on cell membrane, leading to the appearance of pimples, swellings, wrinkles on cell surface of

microorganisms (Wang et al., 2013; Yang et al., 2012) and buds on *Listeria* cell surface (Ritz et al., 2001).

The consequences in cell depend not only on the pressure level, but also on the holding time of the pressure and environmental factors such as temperature, pH, oxygen availability, and the chemical composition of food (or culture media). Therefore high hydrostatic pressure can induce to cell death but also to cell injury (Abe, 2007). If the accumulate damage exceeds the ability of repairing of the cell, death occurs (Rendueles et al., 2011). In the injury state, cells are alive but they develop sensitivity to physical and chemical environments to which the normal cells were resistant (Jasson et al., 2007). If the process does not have the sufficient intensity to inactivate completely the microorganisms, high pressure processing can inflict a sublethal injury in bacteria, including in *L. monocytogenes* (Jofré et al., 2010; Muñoz-Cuevas et al., 2013; Ritz et al., 2006; Yuste et al., 2004). From microbiological hazard analysis standpoint, the presence of sublethal injury is risky because (i) their lack of ability to growth in selective media, named viable-but-not culturable (VBNC) state, leading to overestimation of lethal effect and (ii) their capacity to recover and grow, if the environmental conditions are appropriate (Abe, 2007; Bozoglu et al., 2004; Jasson et al., 2007). Even though, resuscitation of *L. monocytogenes* was detected in an apparent totally inactivated population after high pressure processing during 25 days of storage at 4 and 20 °C (Ritz et al., 2006).

The technology, at industrial levels, is not able to cause lesions directly in DNA of *L. monocytogenes* (Mohamed et al., 2012), but it can affect DNA replication and transcription of the microorganism through an inhibitory action on their enzymes (Huang et al., 2014). The structure of DNA-enzyme complexes, as the DNA can be degraded due to the action of endonucleases not normally in contact with the nucleic acid (Alpas et al., 2003; Dubins et al., 2001). Translation is one of the most cell processes affected by pressure. Ribosomes are pressure sensitive, especially at pressures above 100 MPa, being dissociated, affecting cell viability (Niven et al., 1999). However, the proteomic response to pressure is species dependent (Jofré et al., 2007). The authors reported that protein expression of pressured-treated *L. monocytogenes* was quite

reactive, identifying 23 upregulated proteins related to protein synthesis energy metabolism, DNA replication, repair factors and synthesis of stress proteins. On the other hand, gene expression of *L. monocytogenes* is affected by high hydrostatic pressure (Bowman et al., 2008). After pressurization, a generalized repair response is induced, upregulating genes related to repair cell membrane, septal rings, DNA and ribosomes. The same authors found high pressure suppresses genes associated with energy metabolism and virulence.

One of the identified genetic responses that allow for increasing survival of *Listeria monocytogenes* upon HPP treatment results from induction of the general stress response mediated by sigma B (σ^B) factor (Wells-Bennik et al., 2008). This factor contributes to *L. monocytogenes* (and to other Gram-positive bacteria) response and survival under non-host-associated environmental stress conditions, including acid, osmotic, oxidative, cold and starvation stress (Chaturongakul et al., 2008). Wemekamp-Kamphuis et al. (2004) demonstrated the importance of σ^B factor on response to high hydrostatic pressure of *L. monocytogenes.* The induction of the factor in mutants showed an increase of pressure-resistance compared to the wild-type strain, whereas its deletion made the mutants more susceptible to pressure.

Controlling *L. monocytogenes* in meat products by high hydrostatic pressure

HPP is commercially used mainly as a non-thermal preservation technology for processed foods and RTE meat products with high consumer acceptance, in comparison to other non-thermal decontamination technologies such as ionizing radiation. Since 1998, there are available meat products processed by using this technology (Bajovic et al., 2012; Tonello, 2010). Nowadays, 25 % of the global HPP equipment is currently used to process meat products (raw minced meats, sliced cooked and dry-cured hams, ready meals, poultry cuts and sausages). Among novel food technologies, high hydrostatic pressure has the most potential to continue being used in meat industry according to

food professionals from industry, academia and government around the world (Jermann et al., 2015).

The technology ensures post-packaging inactivation of spoilage microorganisms, extending the shelf-life of RTE meat products (Han et al., 2011). Regarding the control of pathogenic bacteria, the use of high pressure processing for inactivating *Listeria monocytogenes* in RTE meat products is particularly interesting because its high listericidal efficacy without detrimental effects on product's quality (Bover-Cid et al., 2015). The latter led to food safety authorities to enable to the application of the technology for controlling the pathogen specifically in RTE meat products as post-lethality treatment (AESAN, 2005; FSAI, 2005; Health Canada, 2006, n.d.; USDA-FSIS, 2014). The food authorities demand that each product is validated using a challenge test approach or other kind of science-based documentation for guarantee enough inactivation levels. ILSI (2005) stated that 2 log-inactivation could provide a significant margin of safety in products that are infrequently recontaminated with *Listeria monocytogenes.* The need to validate each product is due to the impact of food matrix on the efficacy of the technology.

The mechanisms of pressure-induced inactivation of bacteria in presence of solutes are complex (Georget et al., 2015). Pressure resistance of microorganisms is intimately linked to osmoregulation, as the uptake of compatible solutes such as glycine betaine and L-carnitine generally increase bacterial resistance to pressure (Abe, 2007; Gänzle and Liu, 2015). Although water activity seemed to have a baroprotective effect at low values ($a_w < 0.90$), the nature of the solute is relevant, as well. Koseki and Yamamoto (2007) determined that the baroprotective effect on *L. monocytogenes* differed depending on the chemical nature of solute even at the same water activity. The authors stated saturation of solution would be an accurate parameter of inhibition in terms of HPP-induced inactivation of bacteria. The impact of solutes on high pressure inactivation of microorganisms has to be considered at two levels (Georget et al., 2015). First, low water activity levels partly explains the baroprotection conferred by solutes in complex matrices. However, it is clear that a decrease in water activity contributes to protein stabilization. Secondly, the authors stated that the individual properties of the solute

and its properties in solution might provide an additional protection against pressure induced inactivation of microorganisms.

Strain variability and the control of *L. monocytogenes*

Natural variability of *Listeria monocytogenes*

It was often assumed that the determination and characterization of the strain variation of microorganisms was considered as not necessary because it was equal to or smaller than experimental variability (Whiting and Golden, 2002). However, *L. monocytogenes* exhibit a varied behavior in different biological aspects. The virulence of the pathogen varies significantly among serotypes and strains (Roberts et al., 2005; Roche et al., 2001). *L. monocytogenes* consists of four evolutionary lineages (I, II, III, IV). Each lineage represents distinct ecologic, genetic and phenotypic characteristics, which appear to affect their ability to cause human listeriosis and to be transmitted through foods (Orsi et al., 2011). Lineage I strains are associated to the most human listeriosis outbreaks, while lineage II are common in foods, soil and other environments, and associated to animal listeriosis cases. On the other hand, lineage III and IV strains are rare and predominantly isolated from animal sources. From serological standpoint, the pathogen is classified into 13 serotypes on the basis of somatic and flagellar antigens (Nadon et al., 2001). The vast majority of human listeriosis cases are caused by serotypes 1/2a, 1/2b, and 4b (EFSA and ECDC, 2015). In addition, the survival to gastrointestinal tract and the *in vitro* capacity of cellular invasion, virulence-associated characteristics, were strain-dependent, as well (Barmpalia-Davis et al., 2008; Werbrouck et al., 2009).

Besides virulence and pathogenicity, the growth of the pathogen at different conditions of pH, water activity and temperature demonstrated to be strain-dependent. Begot et al. (1997) found differences in lag time ranged from 4 h to 4 days, when the microorganism grew at 10 °C, pH 7 and water activity 0.96, grouping the strains in five clusters. The authors found no correlation between serotype and growth capacity.

However, lineage seems to be related to how fast *L. monocytogenes* strains can grow in salt stress. Lineage I and III strains grew significantly faster than lineage II at 37 °C, probably due to their adaptation to grow at human body temperature (Bergholz et al., 2010). At cold temperature, strains exhibited different growth rates, but cold tolerance did not seem to be related to lineage, serotype or origin (Arguedas-Villa et al., 2010). Aryani et al. (2015a) determined strain variability explained around one third of total variability of the growth of *L. monocytogenes*. Despite this contribution was similar to biological variability, the integration of strain variability into mathematical models resulted in a more realistic prediction of the growth in food products.

Although the most of *L. monocytogenes* strains are able to form biofilm, this ability varies strain to strain, according to Kadam et al. (2013). The variation is minimized when the strains grow in nutrient-rich media, where biofilm production levels were similar between strains (Nilsson et al., 2011). Both studies demonstrated a strong correlation between serotype and biofilm formation, being serotypes 1/2a and 1/2b the most biofilm producers. The higher ability and serotype relationship could partly explain why some serotypes are more persistent in food processing surfaces as it happens on meat processing surfaces (Martín et al., 2014).

Impact of strain variability on resistance to preservation technologies

L. monocytogenes strains exhibited marked differences in resistance to several preservation technologies. Depending on the strain, the recommended quantities of hypochlorite and sodium carbonate showed not have sufficient efficiency to clean and disinfect *L. monocytogenes* contaminated processing surfaces in dairy industry (Adrião et al., 2008). The latter made necessary to readjust the concentration of each chemical based on the worst case scenario, a high resistant strain. Other compounds used as antimicrobials in food showed similar results. The sensitivity to lactic acid varied considerably among strains and between its isomers (Gravesen et al., 2004). The strain variation in sensitivity to L-lactic acid was larger than to D-lactic acid. The differences between strains in acid stress tolerance seemed to be related to the presence of

ribosomal protein S21 which is not produced by all the strains (Metselaar et al., 2015). Similarly, some strains demonstrated to be resistant to different bacteriocins. Twenty out of 381 *L. monocytogenes* strains were able to grow in media rich in pediocin and bavaricin (Rasch and Knøchel, 1998), and sakacin P (Tessema et al., 2009).

Thermal processing is one of the most used preservation technology to control *Listeria monocytogenes* in food products. Heat resistance of the pathogen is influenced by age of culture, growth conditions, presence of food components and strain evaluated (Doyle et al., 2001). However, strain variability on thermal resistance of *L. monocytogenes* was four and ten times higher than the biological and experimental variability, respectively. The strain variability was higher in treatments at lower temperature (Aryani et al., 2015b).

Regarding non-thermal technologies, the resistance of *L. monocytogenes* exhibited a high strain dependence. The studies done by Lado and Yousef (2003) and Saldaña et al. (2009) showed the lethal effect of pulsed electric fields varies widely among strains, ranging around 3 log CFU/mL of difference between the most resistant and sensitive strains.

The resistance to high hydrostatic pressure has demonstrated to be species-dependent (Alpas et al., 2000; Santillana-Farakos and Zwietering, 2011). In general, Gram-negative bacteria and cells from exponential growth phase are more pressure-sensitive than Gram-positive bacteria and stationary phase cells. Pressure resistance varied within a species, as well. Some strains of *Staphylococcus aureus* (Rodríguez-Calleja et al., 2006), *Escherichia coli* (Benito et al., 1999; Garcia-Hernandez et al., 2015; Liu et al., 2015; Reineke et al., 2014; Robey et al., 2001; Sheen et al., 2015), *Clostridium botulinum* (Ramaswamy et al., 2013), *Campylobacter* spp. (Martínez-Rodriguez and Mackey, 2005) and *Salmonella* spp. (Alpas et al., 1999) have demonstrated to have higher tolerance than others. The first observations about the differences in pressure resistance of *Listeria monocytogenes* were done by Simpson and Gilmour (1997a and 1997b), comparing three strains. The authors noticed that strain variability was affected by processing conditions, pressure and time, and the constituents of the media. Tay et al. (2003) found a widespread range of inactivation between nine strains, which varied

between pressure conditions. Inactivation levels ranged from 1.4 to 4.3 log CFU/mL at 400 MPa, whereas the increase of pressure up to 500 MPa, led to a lethal effect from 3.9 to above 8 log-inactivation. Even at higher pressures, the differences between pressure resistant and sensitive *L. monocytogenes* strains were evident, reaching 5-log difference at 600 MPa for 60 s (Youart et al., 2010).

Process validation and strain variability

Since resistance of *L. monocytogenes* to high hydrostatic pressure and other preservation technologies varies among strains, the selection of them is a relevant decision when designing and conducting challenge test studies (Lianou and Koutsoumanis, 2013). The aim of challenge test studies is to assess the behavior of pathogens in food products or in systems simulating food-related environments. A cocktail of *L. monocytogenes*, minimum three strains, is suggested for use in validation or challenge studies according to the European Commission 2014 Technical Guidance document (Álvarez-Ordóñez et al., 2015). However, five strains are recommended in challenge testing of high pressure processed products (Balasubramaniam et al., 2004). The strains to be included in that cocktails should demonstrate to be pressure-resistant, but not exhibit an unrealistic value (NACMCF, 2010). Other information about the strains such as their origin and their pathogenicity is useful. It is recommended to include strains isolated from the same product or similar to be tested and strains associated with public health concerns (Lianou and Koutsoumanis, 2013). Therefore, a complete characterization of a variety of strains with regards to pressure-resistance and complementary details such as origin and pathogenicity, will provide valuable information for decision making of strain selection.

The knowledge in strain variation of pathogenic bacteria can provide another tool for conducting tests aimed to assess the performance of high hydrostatic pressure: the use of surrogates. A surrogate is defined as an organism or substance used to study the fate of a pathogen in a specific environment (Sinclair et al., 2012). According to the National Advisory Committee on Microbiological Criteria for Foods (NACMCF), the ideal surrogate

should: be non-pathogenic, have similar inactivation kinetics and susceptibility to injury to the pathogen, reproducible preparation of high-density populations, easily enumerated and differentiated, with similar attachment capabilities and genetically stable (NACMCF, 2010). Surrogates are often released into the environment to assess decontamination or disinfection in water treatment and food processing (Sinclair et al., 2012), in those cases for testing equipment and processes in-plant, when the introduction of the pathogen poses a risk (NACMCF, 2010).

The ultimate goal of selecting a sufficiently representative surrogate is to improve public health through a health-based risk assessment framework (Sinclair et al., 2012). Then, the inclusion of non-pathogenic bacteria in the strain set to verify if their inactivation kinetics and susceptibility is closed to that exhibited by the strains of the pathogen of concern could be useful to find potential and appropriate surrogates. Following this approach, Garcia-Hernandez et al. (2015) designed and validated a cocktail of non-pathogenic *Escherichia coli* to evaluate lethal effect of high hydrostatic pressure on verotoxigenic *E. coli* (VTEC). The proposed cocktail comprised of five non-pathogenic *Escherichia coli* strains had a comparable inactivation and sublethal injury to a five VTEC strains cocktail when both were challenged at 600 MPa for 30 min at 20 °C in ground beef. Similar works have been performed in *Escherichia coli* recently (Liu et al., 2015; Reineke et al., 2014).

In the case of *Listeria monocytogenes, Listeria innocua* has been proposed to be used as its surrogate in several food processing technologies (Friedly et al., 2008; Ma et al., 2007; Uesugi and Moraru, 2009). From phylogenetic standpoint, *Listeria innocua* and *L. monocytogenes* are close species, evolving *L. innocua* from the ancestor to the *L. monocytogenes* serogroup 4 (Den Bakker et al., 2010; Doumith et al., 2004). Traditionally, the ecological cohabitation, genomic synteny, and physiological similarity of the two species have supported the use of *L. innocua* for predicting the behavior of *Listeria monocytogenes* in farm and food processing environments (Milillo et al., 2012). Despite the closeness from genetic standpoint between both species (Glaser et al., 2001), the response of *Listeria monocytogenes* and *Listeria innocua* to different environmental stresses could be distinct. Transcriptomic studies demonstrated that salt

stress induces 168 σ^B -dependent genes were positively regulated in *L. monocytogenes*, whereas in *L. innocua,* only 65 genes are induced (Raengpradub et al., 2008). The same authors found that both species differ in σ^B –dependent acid stress resistance and have species-specific σ^B –dependent, as well. This different gene expression at stressful conditions could lead to a variable response depending on the type and intensity of the adverse condition.

Some strains of *L. innocua* have demonstrated to be a suitable surrogate of *Listeria monocytogenes* in thermal processing of meat products (Friedly et al., 2008; Kamat and Nair, 1996; Mackey et al., 1990; O'Bryan et al., 2006). Its suitability as surrogate in preservation of meat products by pulsed light technology has been validated, as well (Lasagabaster and de Marañón, 2012; Uesugi and Moraru, 2009). However, the use of *L. innocua* as surrogate in studies about decontamination by radiation has been no conclusive. While the studies of Kamat and Nair (1996) and Hoz et al. (2008) showed the behavior of the non-pathogenic species was similar to *L. monocytogenes* after radiation, other studies found significant differences between the inactivation levels caused by radiation between both *Listeria* genus species, suggesting to use other non-pathogenic microorganisms as surrogates (Niemira, 2010; Rodriguez et al., 2006). The resistance of *L. innocua* to citral, an antimicrobial terpenoid, was similar to that exhibited by the pathogenic bacteria. Both species grew and were injured at comparable levels (Silva-Angulo et al., 2015). However, when both microorganisms were exposed to carvacrol, *L. monocytogenes* grew faster in presence of this antimicrobial compound than the non-pathogenic bacterium, compromised its use as surrogate in these conditions (Silva-Angulo et al., 2014).

Although *L. innocua* has been used as surrogate in studies about the control of *L. monocytogenes* by high hydrostatic pressure in meat products (Carlez et al., 1993; Escriu and Mor-Mur, 2009; Merialdi et al., 2015; Vercammen et al., 2011), the studies that compare the resistance to pressure of both microorganisms are not conclusive. Tay et al. (2003) compared the inactivation levels of *L. innocua* to nine strains of *L. monocytogenes* at different pressures, and stated that the nonpathogenic species presented an intermediate pressure-resistance. Similar results were obtained by Waite-

Cusic et al. (2011), who suggested the use of a strain *Lactobacillus plantarum* as surrogate for high pressure processing and pulsed electric field of whey instead of *L. innocua.* However, *L. innocua* exhibited a similar inactivation levels to *L. monocytogenes* in high pressure-processed yogurt (Evrendilek and Balasubramaniam, 2011). Santillana-Farakos and Zwietering (2011) found no significant differences in log D_P – values, from published data, between both *Listeria* genus species, regardless the strain, substrate or stage of growth of the cells. Although *Listeria innocua* seems to be a suitable surrogate of *L. monocytogenes,* the scientific evidence suggests that this statement should be validated case-to-case, because the preservation technology (and its intensity), strains and food matrix can influence on the comparison between both species.

High pressure processing has demonstrated to be an efficient preservation technology for controlling spoilage and pathogenic microorganisms, such as *Listeria monocytogenes* in ready-to-eat meat products. However, the genetic and phenotypic diversity of this bacterium allow it to resist to adverse environmental conditions. Understanding the influence of strain variability on the resistance to high hydrostatic pressure will be essential to design safer high pressure-processed meat products.

References

Abe, F., 2007. Exploration of the effects of high hydrostatic pressure on microbial growth, physiology and survival: perspectives from piezophysiology. *Biosci. Biotechnol. Biochem.* 71, 2347–2357.

Abu Mraheil, M. 2013. Listeria, in: Rosenberg, E., DeLong, E.F., Stackebrandt, E., Thompson, F., Lory, S. (Eds.), The Prokaryotes - Human Microbiology. Springer-Verlag Berlin Heidelberg, pp. 219–235.

Adrião, A., Vieira, M., Fernandes, I., Barbosa, M., Sol, M., Tenreiro, R.P., Chambel, L., Barata, B., Zilhao, I., Shama, G., Perni, S., Jordan, S.J., Andrew, P.W., Faleiro, M.L., 2008. Marked intra-strain variation in response of *Listeria monocytogenes* dairy isolates to acid or salt stress and the effect of acid or salt adaptation on adherence to abiotic surfaces. *Int. J. Food Microbiol.* 123, 142–150.

AESAN, 2005. Opinión del Comité científico de la AESAN sobre una cuestión presentada por la Dirección Ejecutiva, en relación con la aplicación de altas presiones en carne y productos cárnicos (Ref. AESAN-2003-007). Rev. del Com. Científico la AESAN 1, 26–71.

Ahn, D.U., Lee, E.J., 2012. Food Irradiation Research and Technology, Food Irradiation Research and Technology: Second Edition. John Wiley & Sons, Inc., Hoboken, NJ, USA.

Alpas, H., Kalchayanand, N., Bozoglu, F., Ray, B., 2000. Interactions of high hydrostatic pressure, pressurization temperature and pH on death and injury of pressure-resistant and pressure-sensitive strains of foodborne pathogens. *Int. J. Food Microbiol*. 60, 33–42.

Alpas, H., Kalchayanand, N., Bozoglu, F., Sikes, A., Dunne, C.P., Ray, B., 1999. Variation in resistance to hydrostatic pressure among strains of food-borne pathogens. *Appl. Environ. Microbiol*. 65, 4248–4251.

Alpas, H., Lee, J., Bozoglu, F., Kaletunç, G., 2003. Evaluation of high hydrostatic pressure sensitivity of *Staphylococcus aureus* and *Escherichia coli* O157:H7 by differential scanning calorimetry. *Int. J. Food Microbiol*. 87, 229–237.

Álvarez-Ordóñez, A., Leong, D., Hickey, B., Beaufort, A., Jordan, K., 2015. The challenge of challenge testing to monitor *Listeria monocytogenes* growth on ready-to-eat foods in Europe by following the European Commission (2014) Technical Guidance document. *Food Res. Int.* 75, 233–243.

Arguedas-Villa, C., Stephan, R., Tasara, T., 2010. Evaluation of cold growth and related gene transcription responses associated with *Listeria monocytogenes* strains of different origins. *Food Microbiol*. 27, 653–660.

Aryani, D.C., den Besten, H.M.W., Hazeleger, W.C., Zwietering, M.H., 2015a. Quantifying strain variability in modeling growth of *Listeria monocytogenes*. *Int. J. Food Microbiol*. 208, 19–29.

Aryani, D.C., den Besten, H.M.W., Hazeleger, W.C., Zwietering, M.H., 2015b. Quantifying variability on thermal resistance of *Listeria monocytogenes*. *Int. J. Food Microbiol*. 193, 130–138.

Baer, A.A., Miller, M.J., Dilger, A.C., 2013. Pathogens of interest to the pork industry: a review of research on interventions to assure food safety. *Compr. Rev. Food Sci. Food Saf*. 12, 183–217.

Bajovic, B., Bolumar, T., Heinz, V., 2012. Quality considerations with high pressure processing of fresh and value added meat products. *Meat Sci*. 92, 280–9.

Balasubramaniam, V.M., Ting, E.Y., Stewart, C.M., Robbins, J. a., 2004. Recommended laboratory practices for conducting high-pressure microbial inactivation experiments. *Innov. Food Sci. Emerg. Technol*. 5, 299–306.

Barmpalia-Davis, I.M., Geornaras, I., Kendall, P. a., Sofos, J.N., 2008. Differences in survival among 13 *Listeria monocytogenes* strains in a dynamic model of the stomach and small intestine. *Appl. Environ. Microbiol*. 74, 5563–5567.

Begot, C., Lebert, I., Lebert, A., 1997. Variability of the response of 66 *Listeria monocytogenes* and *Listeria innocua* strains to different growth conditions. *Food Microbiol*. 14, 403–412.

Benito, A., Ventoura, G., Casadei, M., Robinson, T., Mackey, B., 1999. Variation in resistance of natural isolates of *Escherichia coli* O157 to high hydrostatic pressure, mild heat, and other stresses. *Appl. Environ. Microbiol*. 65, 1564–1569.

Bergholz, T.M., den Bakker, H.C., Fortes, E.D., Boor, K.J., Wiedmann, M., 2010. Salt stress phenotypes in *Listeria monocytogenes* vary by genetic lineage and temperature. *Foodborne Pathog. Dis*. 7, 1537–1549.

Bermúdez-Aguirre, D., Barbosa-Cánovas, G. V., 2011. An Update on High Hydrostatic Pressure, from the Laboratory to Industrial Applications. *Food Eng. Rev*. 3, 44–61.

Bourdichon, F., 2014. *Listeria monocytogenes*: what have we learned from the last 30 years? *New food Mag. Food Saf. Suppl*. 17, 3–7.

Bover-Cid, S., Belletti, N., Aymerich, T., Garriga, M., 2015. Modeling the protective effect of a_w and fat content on the high pressure resistance of *Listeria monocytogenes* in dry-cured ham. *Food Res. Int*. 75, 194–199.

Bover-Cid, S., Belletti, N., Garriga, M., Aymerich, T., 2011. Model for *Listeria monocytogenes* inactivation on dry-cured ham by high hydrostatic pressure processing. *Food Microbiol*. 28, 804–809.

Bowman, J.P., Bittencourt, C.R., Ross, T., 2008. Differential gene expression of *Listeria monocytogenes* during high hydrostatic pressure processing. *Microbiology* 154, 462–475.

Bozoglu, F., Alpas, H., Kaletunç, G., 2004. Injury recovery of foodborne pathogens in high hydrostatic pressure treated milk during storage. *FEMS Immunol. Med. Microbiol*. 40, 243–7.

Campus, M., 2010. High Pressure Processing of Meat, Meat Products and Seafood. *Food Eng. Rev*. 2, 256–273.

Carlez, A., Rosec, J., Richard, N., Cheftel, J., 1993. High Pressure Inactivation of *Citrobacter freundii*, *Pseudomonas fluorescens* and *Listeria innocua* in Inoculated Minced Beef Muscle. *LWT - Food Sci. Technol*. 26, 357–363.

Chaturongakul, S., Raengpradub, S., Wiedmann, M., Boor, K.J., 2008. Modulation of stress and virulence in *Listeria monocytogenes*. *Trends Microbiol*. 16, 388–396.

Chenal-Francisque, V., Lopez, J., Cantinelli, T., Caro, V., Tran, C., Leclercq, A., Lecuit, M., Brisse, S., 2011. Worldwide distribution of major clones of *Listeria monocytogenes*. *Emerg. Infect. Dis*. 17, 1110–1112.

Considine, K.M., Kelly, A.L., Fitzgerald, G.F., Hill, C., Sleator, R.D., 2008. High-pressure processing- effects on microbial food safety and food quality. *FEMS Microbiol. Lett*. 281, 1–9.

Crim, S.M., Iwamoto, M., Huang, J.Y., Griffin, P.M., Gilliss, D., Cronquist, A.B., Cartter, M., Tobin-D'Angelo, M., Blythe, D., Smith, K., Lathrop, S., Zansky, S., Cieslak, P.R., Dunn, J., Holt, K.G., Lance, S., Tauxe, R., Henao, O.L., 2014. Incidence and trends of infection with pathogens transmitted commonly through food--Foodborne Diseases Active Surveillance Network, 10 U.S. sites, 2006-2013. *MMWR. Morb. Mortal. Wkly*. Rep. 63, 328–32.

Cruz, J., 2013. Los productores de jamón curado buscan suplir con las exportaciones la caída de las ventas en España. *Eurocarne* 35–44.

de Noordhout, C.M., Devleesschauwer, B., Angulo, F.J., Verbeke, G., Haagsma, J., Kirk, M., Havelaar, A., Speybroeck, N., 2014. The global burden of listeriosis: a systematic review and meta-analysis. *Lancet Infect. Dis*. 14, 1073–1082.

Den Bakker, H.C., Bundrant, B.N., Fortes, E.D., Orsi, R.H., Wiedmann, M., 2010. A population genetics-based and phylogenetic approach to understanding the evolution of virulence in the genus *Listeria*. *Appl. Environ. Microbiol*. 76, 6085–6100.

Doumith, M., Buchrieser, C., Glaser, P., Jacquet, C., Martin, P., 2004. Differentiation of the Major *Listeria monocytogenes* Serovars by Multiplex PCR. *J. Clin. Microbiol*. 42, 3819–3822.

Doyle, M.E., Mazzotta, A.S., Wang, T., Wiseman, D.W., Scott, V.N., 2001. Heat Resistance of *Listeria monocytogenes. J. Food Prot*. 64, 410–429.

Dubins, D.N., Lee, A., Macgregor, R.B., Chalikian, T. V., 2001. On the Stability of Double Stranded Nucleic Acids. *J. Am. Chem. Soc.* 123, 9254–9259.

EFSA, ECDC, 2015. Trends and sources of zoonoses, zoonotic agents and food-borne. *EFSA J.* 13, 1–162.

Escriu, R., Mor-Mur, M., 2009. Role of quantity and quality of fat in meat models inoculated with *Listeria innocua* or *Salmonella Typhimurium* treated by high pressure and refrigerated stored. *Food Microbiol*. 26, 834–40.

Evrendilek, G.A., Balasubramaniam, V.M., 2011. Inactivation of *Listeria monocytogenes* and *Listeria innocua* in yogurt drink applying combination of high pressure processing and mint essential oils. *Food Control* 22, 1435–1441.

FAO, 2004. Risk assessment of *Listeria monocytogenes* in ready-to-eat foods: Technical report, Microbiology risk assesment series 5. Geneva, Switzerland.

Farkas, D.F., Hoover, D.G., 2000. High pressure processing. *J. Food Sci*. 65, 47–64.

FDA, 2000. Kinetics of microbial inactivation for alternative food processing technologies: High pressure procesing, *Safe Practices for Food Processes*.

Friedly, E.C., Crandall, P.G., Ricke, S., O'Bryan, C. A., Martin, E.M., Boyd, L.M., 2008. Identification of *Listeria innocua* surrogates for *Listeria monocytogenes* in hamburger patties. *J. Food Sci*. 73, 174–178.

FSAI, 2005. The Control and Management of *Listeria monocytogenes* Contamination of Food. Dublin.

Gänzle, M., Liu, Y., 2015. Mechanisms of pressure-mediated cell death and injury in Escherichia coli: from fundamentals to food applications*. Front. Microbiol.* 6, 1–10.

Garcia-Hernandez, R., Mcmullen, L., Gänzle, M.G., 2015. Development and validation of a surrogate strain cocktail to evaluate bactericidal effects of pressure on verotoxigenic *Escherichia coli. Int. J. Food Microbiol*. 205, 16–22.

Georget, E., Sevenich, R., Reineke, K., Mathys, A., Heinz, V., Callanan, M., Rauh, C., Knorr, D., 2015. Inactivation of microorganisms by high isostatic pressure processing in complex matrices : A review. *Innov. Food Sci. Emerg. Technol*. 27, 1–14.

Glaser, P., Frangeul, L., Buchrieser, C., Rusniok, C., Amend, A., Baquero, F., Berche, P., Bloecker, H., Brandt, P., Chakraborty, T., Charbit, A., Chetouani, F., Couvé, E., de Daruvar, A., Dehoux, P., Domann, E., Domínguez-Bernal, G., Duchaud, E., Durant, L., Dussurget, O., Entian, K.D., Fsihi, H., García-del Portillo, F., Garrido, P., Gautier, L., Goebel, W., Gómez-López, N., Hain, T., Hauf, J., Jackson, D., Jones, L.M., Kaerst, U., Kreft, J., Kuhn, M., Kunst, F., Kurapkat, G., Madueno, E., Maitournam, A., Vicente, J.M., Ng, E., Nedjari, H., Nordsiek, G., Novella, S., de Pablos, B., Pérez-Diaz, J.C., Purcell, R., Remmel, B., Rose, M., Schlueter, T., Simoes, N., Tierrez, A., Vázquez-Boland, J.A., Voss, H., Wehland, J., Cossart, P., 2001. Comparative genomics of *Listeria* species. *Science* 294, 849–52.

Gravesen, A., Diao, Z., Voss, J., Budde, B.B., Knøchel, S., 2004. Differential inactivation of *Listeria monocytogenes* by D- and L-lactic acid. Lett. Appl. Microbiol. 39, 528–532.

Guillet, C., Join-Lambert, O., Le Monnier, A., Leclercq, A., Mechaï, F., Mamzer-Bruneel, M.F., Bielecka, M.K., Scortti, M., Disson, O., Berche, P., Vazquez-Boland, J., Lortholary, O., Lecuit, M., 2010. Human listeriosis caused by *Listeria ivanovii. Emerg. Infect. Dis.* 16, 136–8.

Han, Y., Jiang, Y., Xu, X., Sun, X., Xu, B., Zhou, G., 2011. Effect of high pressure treatment on microbial populations of sliced vacuum-packed cooked ham. *Meat Sci.* 88, 682–8.

Health Canada, 2011. Policy on *Listeria monocytogenes* in Ready-to-Eat Foods.

Health Canada, 2006. Use of High Hydrostatic Pressure for the Control of *L. monocytogenes* in Ready to Eat (RTE) Meats and Poultry. Nov. Food Inf. URL http://www.hc-sc.gc.ca/fn-an/gmf-agm/appro/nf-an138decdoc-eng.php (accessed 8.20.15).

Health Canada, 2010. Guidance for Industry on Novelty Determination of High Pressure Processing (HPP) -Treated Food Products.

Hoffmann, S., Maculloch, B., Batz, M., 2015. Economic Burden of Major Foodborne Illnesses Acquired in the United States. Washington D. C.

Hoz, L., Cambero, M.I., Cabeza, M.C., Herrero, A.M., Ordóñez, J.A., 2008. Elimination of *Listeria monocytogenes* from vacuum-packed dry-cured ham by E-beam radiation. *J. Food Prot*. 71, 2001–2006.

Huang, H.W., Lung, H.M., Yang, B.B., Wang, C.Y., 2014. Responses of microorganisms to high hydrostatic pressure processing. *Food Control* 40, 250–259.

Hugas, M., 2003. Functionalty of *enterococci* in meat products. *Int. J. Food Microbiol.* 88, 223–233.

ILSI, 2005. Achieving continuous improvement in reductions in foodborne listeriosis--a risk-based approach. *J. Food Prot.* 68, 1932–1994.

Ivanek, R., Gröhn, Y.T., Tauer, L.W., Wiedmann, M., 2004. The cost and benefit of *Listeria monocytogenes* food safety measures. *Crit. Rev. Food Sci. Nutr.* 44, 513–523.

Jacobsen, T., Budde, B.B., Koch, A.G., 2003. Application of *Leuconostoc carnosum* for biopreservation of cooked meat products. *J. Appl. Microbiol*. 95, 242–249.

Jasson, V., Uyttendaele, M., Rajkovic, A., Debevere, J., 2007. Establishment of procedures provoking sub-lethal injury of *Listeria monocytogenes*, *Campylobacter jejuni* and *Escherichia coli* O157 to serve method performance testing. *Int. J. Food Microbiol*. 118, 241–249.

Jermann, C., Koutchma, T., Margas, E., Leadley, C., Ros-Polski, V., 2015. Mapping trends in novel and emerging food processing technologies around the world. *Innov. Food Sci. Emerg. Technol*. 31, 14–27.

Jiang, J., Xiong, Y.L., 2014. Technologies and Mechanisms for Safety Control of Ready-to-Eat Muscle Foods: An Updated Review*. Crit. Rev. Food Sci. Nutr*. 55:13, 1886-1901.

Jofré, A., Aymerich, T., Bover-Cid, S., Garriga, M., 2010. Inactivation and recovery of *Listeria monocytogenes*, *Salmonella enterica* and *Staphylococcus aureus* after high hydrostatic pressure treatments up to 900 MPa. *Int. Microbiol*. 13, 105–112.

Jofré, A., Champomier-Vergès, M., Anglade, P., Baraige, F., Martín, B., Garriga, M., Zagorec, M., Aymerich, T., 2007. Protein synthesis in lactic acid and pathogenic

bacteria during recovery from a high pressure treatment. *Res. Microbiol*. 158, 512–520.

Kadam, S.R., den Besten, H.M.W., van der Veen, S., Zwietering, M.H., Moezelaar, R., Abee, T., 2013. Diversity assessment of *Listeria monocytogenes* biofilm formation: Impact of growth condition, serotype and strain origin. *Int. J. Food Microbiol*. 165, 259–264.

Kamat, A. S., Nair, P.M., 1996. Identification of *Listeria innocua* as a Biological Indicator for Inactivation of *L. monocytogenes* by some Meat Processing Treatments. *LWT - Food Sci. Technol.* 720, 714–720.

Koseki, S., Yamamoto, K., 2007. Water activity of bacterial suspension media unable to account for the baroprotective effect of solute concentration on the inactivation of Listeria monocytogenes by high hydrostatic pressure. *Int. J. Food Microbiol*. 115, 43–47.

Lado, B.H., Yousef, A.E., 2003. Selection and Identification of a *Listeria monocytogenes* Target Strain for Pulsed Electric Field Process Optimization. *Appl. Environ. Microbiol*. 69.

Lasagabaster, A., de Marañón, I.M., 2012. Sensitivity to pulsed light technology of several spoilage and pathogenic bacteria isolated from fish products. *J. Food Prot*. 75, 2039–44.

Lianou, A., Koutsoumanis, K.P., 2013. Strain variability of the behavior of foodborne bacterial pathogens: A review. *Int. J. Food Microbiol*. 167, 310–321.

Liu, Y., Gill, A., Mcmullen, L., Gänzle, M.G., 2015. Variation in Heat and Pressure resistance of Verotoxigenic and Nontoxigenic *Escherichia coli*. *J. Food Prot*. 78, 111–120.

Luber, P., 2011. The *Codex Alimentarius* guidelines on the application of general principles of food hygiene to the control of *Listeria monocytogenes* in ready-to-eat foods. *Food Control* 22, 1482–1483.

Lundén, J.M., Autio, T.J., Sjöberg, A.M., Korkeala, H.J., 2003. Persistent and Nonpersistent *Listeria monocytogenes* Contamination in Meat and Poultry Processing Plants. *J. Food Prot.* 66, 2062–2069.

Lunden, J.M., Miettinen, M.K., Autio, T.J., Korkeala, H.J., 2000. Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surface after short contact times. *J. Food Prot*. 63, 1204–1207.

Ma, L., Kornacki, J.L., Zhang, G., Lin, C.M., Doyle, M.P., 2007. Development of thermal surrogate microorganisms in ground beef for in-plant critical control point validation studies. *J. Food Prot*. 70, 952–957.

Mackey, B.M., Pritchet, C., Norris, A., Mead, G.C., 1990. Heat resistance of *Listeria*: Strain differences and effects of meat type and curing salts. *Lett. Appl. Microbiol*. 10, 251–255.

Mangen, M.-J.J., Bouwknegt, M., Friesema, I.H.M., Haagsma, J. a., Kortbeek, L.M., Tariq, L., Wilson, M., van Pelt, W., Havelaar, A.H., 2015. Cost-of-illness and disease burden of food-related pathogens in The Netherlands, 2011. *Int. J. Food Microbiol*. 196, 84–93.

Martín, B., Perich, A., Gómez, D., Yangüela, J., Rodríguez, A., Garriga, M., Aymerich, T., 2014. Diversity and distribution of *Listeria monocytogenes* in meat processing plants. *Food Microbiol.* 44, 119–127.

Martínez-Rodriguez, a., Mackey, B.M., 2005. Factors affecting the pressure resistance of some *Campylobacter* species. *Lett. Appl. Microbiol*. 41, 321–326.

Merialdi, G., Ramini, M., Ravanetti, E., Gherri, G., Bonilauri, P., 2015. Reduction of *Listeria innocua* contamination in vacuum-packaged dry-cured Italian pork products after high hydrostatic pressure treatment. *Ital. J. Food Saf*. 4, 101–103.

Metselaar, K.I., den Besten, H.M.W., Boekhorst, J., van Hijum, S. a. F.T., Zwietering, M.H., Abee, T., 2015. Diversity of acid stress resistant variants of *Listeria monocytogenes* and the potential role of ribosomal protein S21 encoded by *rpsU*. *Front. Microbiol*. 6, 1–12.

Milillo, S.R., Friedly, E.C., Saldivar, J.C., Muthaiyan, A., O'Bryan, C., Crandall, P.G., Johnson, M.G., Ricke, S.C., 2012. A review of the ecology, genomics, and stress response of *Listeria innocua* and *Listeria monocytogenes*. *Crit. Rev. Food Sci. Nutr*. 52, 712–25.

Mohamed, H.M.H., Diono, B.H.S., Yousef, A.E., 2012. Structural changes in *Listeria monocytogenes* treated with gamma radiation, pulsed electric field and ultra-high pressure. *J. Food Saf.* 32, 66–73.

Moore, T., 2015. The South's beloved Blue Bell ice cream is finally getting good news Washington Post. http://www.washingtonpost.com/news/wonkblog/wp/2015/07/17/the-souths-beloved-blue-bell-ice-cream-is-finally-getting-good-news/ (accessed 10.27.15).

Muñoz-Cuevas, M., Guevara, L., Aznar, A., Martínez, A., Periago, P.M., Fernández, P.S., 2013. Characterisation of the resistance and the growth variability of *Listeria monocytogenes* after high hydrostatic pressure treatments. *Food Control* 29, 409–415.

Muriana, P.M., Quimby, W., Davidson, C.A., Grooms, J., 2002. Postpackage pasteurization of ready-to-eat deli meats by submersion heating for reduction of *Listeria monocytogenes*. *J. Food Prot*. 65, 963–969.

Murphy, R.Y., Hanson, R.E., Johnson, N.R., Scott, L.L., Feze, N., Chappa, K., 2005. Combining antimicrobial and steam treatments in a vacuum-packaging system to control *Listeria monocytogenes* on ready-to-eat franks. *J. Food Sci.* 70. 234-241.

NACMCF, 2010. Parameters for determining inoculated pack/challenge study protocols. *J. Food Prot.* 73, 140–202.

Nadon, C. a., Woodward, D.L., Young, C., Rodgers, F.G., Wiedmann, M., 2001. Correlations between molecular subtyping and serotyping of *Listeria monocytogenes*. *J. Clin. Microbiol*. 39, 2704–2707.

Németh, C., Castillo, L.A., Horváth, F., Zeke, I., Friedrich, L., Balla, C., Németh, Z., Póti, P., 2015. High hydrostatic pressure: Can we trust published data? *Acta Univ. Sapientiae*, Aliment. 8, 86–94.

Niemira, B. a., 2010. Irradiation sensitivity of planktonic and biofilm-associated *Listeria monocytogenes* and *L. innocua* as influenced by temperature of biofilm formation. *Food Bioprocess Technol.* 3, 257–264.

Nilsson, R.E., Ross, T., Bowman, J.P., 2011. Variability in biofilm production by *Listeria monocytogenes* correlated to strain origin and growth conditions. *Int. J. Food Microbiol*. 150, 14–24.

Niven, G.W., Miles, C.A., Mackey, B.M., 1999. The effects of hydrostatic pressure on ribosome conformation in *Escherichia coli*: An in vivo study using differential scanning calorimetry. *Microbiology* 145, 419–425.

NSW food authority, 2013. Survey of *Listeria monocytogenes* in sliced pre-packaged RTE meats. Australia.

O'Bryan, C., Crandall, P.G., Martin, E.M., Griffis, C.L., Johnson, M.G., 2006. Heat resistance of *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* 0157:H7, and *Listeria innocua* M1, a potential surrogate for *Listeria monocytogenes*, in meat and poultry: A Review. *J. Food Sci.* 71, 23–30.

Orsi, R.H., Bakker, H.C.D., Wiedmann, M., 2011. *Listeria monocytogenes* lineages: Genomics, evolution, ecology, and phenotypic characteristics. *Int. J. Med. Microbiol*. 301, 79–96.

Pérez-Rodríguez, F., Castro, R., Posada-Izquierdo, G.D., Valero, A., Carrasco, E., García-Gimeno, R.M., Zurera, G., 2010. Evaluation of hygiene practices and microbiological quality of cooked meat products during slicing and handling at retail. *Meat Sci*. 86, 479–485.

Pirie, J.H.H., 1940. *Listeria*: Change of name for a genus of bacteria. *Nature*, 145, 264-270.

Raengpradub, S., Wiedmann, M., Boor, K.J., 2008. Comparative analysis of the sigma B-dependent stress responses in *Listeria monocytogenes* and *Listeria innocua* strains exposed to selected stress conditions. *Appl. Environ. Microbiol*. 74, 158–71.

Ramaswamy, H.S., Shao, Y., Bussey, J., Austin, J., 2013. Screening of twelve *Clostridium botulinum* (group I) spores for high-pressure resistance at elevated-temperatures. *Food Bioprod. Process*. 91, 403–412.

Rasch, M., Knøchel, S., 1998. Variations in tolerance of *Listeria monocytogenes* to nisin, pediocin PA-1 and bavaricin A. *Lett. Appl. Microbiol*. 27, 275–278.

RASFF, 2015. RASFF Portal [WWW Document]. Rapid Alert Syst. Food Feed. URL https://webgate.ec.europa.eu/rasff-window/portal/ (accessed 10.17.15).

Reineke, K., Sevenich, R., Hertwig, C., Janßen, T., Fröhling, A., Knorr, D., Wieler, L.H., Schlüter, O., 2014. Comparative study on the high pressure inactivation behavior of the Shiga toxin-producing *E. coli* O104:H4 and O157:H7 outbreak strains and a non-pathogenic surrogate. *Food Microbiol*. 46, 184–194.

Rendueles, E., Omer, M.K., Alvseike, O., Alonso-Calleja, C., Capita, R., Prieto, M., 2011. Microbiological food safety assessment of high hydrostatic pressure processing: A review. *LWT - Food Sci. Technol.* 44, 1251–1260.

Ritz, M., Pilet, M.F., Jugiau, F., Rama, F., Federighi, M., 2006. Inactivation of *Salmonella Typhimurium* and *Listeria monocytogenes* using high-pressure treatments: Destruction or sublethal stress? *Lett. Appl. Microbiol*. 42, 357–362.

Ritz, M., Tholozan, J.. L., Federighi, M., Pilet, M. F., 2002. Physiological damages of *Listeria monocytogenes* treated by high hydrostatic pressure. *Int. J. Food Microbiol*. 79, 47–53.

Ritz, M., Tholozan, J.L., Federighi, M., Pilet, M.F., 2001. Morphological and Physiological Characterization of *Listeria monocytogenes* Subjected to High Hydrostatic Pressure Morphological and Physiological Characterization of Listeria monocytogenes Subjected to High Hydrostatic Pressure. *Appl. Environ. Microbiol*. 67, 2240–2247.

Roberts, A., Chan, Y., Wiedmann, M., 2005. Definition of genetically distinct attenuation mechanisms in naturally virulence-attenuated *Listeria monocytogenes* by comparative cell culture and molecular characterization. *Appl. Environ. Microbiol*. 71, 3900–3910.

Robey, M., Benito, A., Hutson, R.H., Pascual, C., Park, S.F., Mackey, B.M., 2001. Variation in resistance to high hydrostatic pressure and *rpoS* heterogeneity in natural isolates of *Escherichia coli* O157: H7. *Appl. Environ. Microbiol*. 67, 4901-4907.

Roche, S.M., Velge, P., Bottreau, E., Durier, C., Marquet-van der Mee, N., Pardon, P., 2001. Assessment of the virulence of *Listeria monocytogenes*: Agreement between

a plaque-forming assay with HT-29 cells and infection of immunocompetent mice. *Int. J. Food Microbiol.* 68, 33–44.

Rodriguez, O., Castell-Perez, M.E., Ekpanyaskun, N., Moreira, R.G., Castillo, A., 2006. Surrogates for validation of electron beam irradiation of foods. *Int. J. Food Microbiol*. 110, 117–122.

Rodríguez-Calleja, J.M., Cebrián, G., Condón, S., Mañas, P., 2006. Variation in resistance of natural isolates of *Staphylococcus aureus* to heat, pulsed electric field and ultrasound under pressure. *J. Appl. Microbiol*. 100, 1054–1062.

Saldaña, G., Puértolas, E., López, N., García, D., Álvarez, I., Raso, J., 2009. Comparing the PEF resistance and occurrence of sublethal injury on different strains of *Escherichia coli*, *Salmonella Typhimurium*, *Listeria monocytogenes* and *Staphylococcus aureus* in media of pH 4 and 7. *Innov. Food Sci. Emerg. Technol*. 10, 160–165.

Santillana-Farakos, S.M., Zwietering, M.H., 2011. Data analysis of the inactivation of foodborne microorganisms under high hydrostatic pressure to establish global kinetic parameters and influencing factors. *J. Food Prot*. 74, 2097–2106.

Sauders, B.D., Overdevest, J., Fortes, E., Windham, K., Schukken, Y., Lembo, A., Wiedmann, M., 2012. Diversity of *Listeria* species in urban and natural environments. *Appl. Environ. Microbiol*. 78, 4420–33.

Sheen, S., Cassidy, J., Scullen, B., Sommers, C., 2015. Inactivation of a diverse set of Shiga toxin-producing *Escherichia coli* in ground beef by high pressure processing. *Food Microbiol*. 52, 84–87.

Silva-Angulo, A.B., Zanini, S.F., Rodrigo, D., Rosenthal, A., Martinez, A., 2014. Growth kinetics of *Listeria innocua* and *Listeria monocytogenes* under exposure to carvacrol and the occurrence of sublethal damage. *Food Control* 37, 336–342.

Silva-Angulo, A.B., Zanini, S.F., Rosenthal, A., Rodrigo, D., Klein, G., Martínez, A., 2015. Comparative Study of the Effects of Citral on the Growth and Injury of *Listeria innocua* and *Listeria monocytogenes* Cells. *PLoS One* 10.

Simpson, R., Gilmour, A., 1997a. The resistance of *Listeria monocytogenes* to high hydrostatic pressure in foods. *Food Microbiol*. 14, 567–573.

Simpson, R., Gilmour, A., 1997b. The effect of high hydrostatic pressure on *Listeria monocytogenes* in phosphate-buffered saline and model food systems. *J. Appl. Microbiol*. 83, 181–188.

Sinclair, R.G., Rose, J.B., Hashsham, S. a., Gerba, C.P., Haase, C.N., 2012. Criteria for selection of surrogates used to study the fate and control of pathogens in the environment. *Appl. Environ. Microbiol*. 78, 1969–1977.

Swaminathan, B., Gerner-Smidt, P., 2007. The epidemiology of human listeriosis. *Microbes Infect.* 9, 1236–1243.

Tay, A., Shellhammer, T.H., Yousef, A.E., Chism, G.W., 2003. Pressure death and tailing behavior of *Listeria monocytogenes* strains having different barotolerances. *J. Food Prot*. 66, 2057–2061.

Tessema, G.T., Møretrø, T., Kohler, A., Axelsson, L., Naterstad, K., 2009. Complex phenotypic and genotypic responses of *Listeria monocytogenes* strains exposed to the class IIa bacteriocin sakacin P. *Appl. Environ. Microbiol*. 75, 6973–6980.

The Commission of the European Communities, 2005. Commission regulation (EC No 2073/2005) of 15 November 2005 on microbial criteria for foodstuffs. *Off. J. Eur. Union* L338, 1–26.

Thévenot, D., Dernburg, A., Vernozy-Rozand, C., 2006. An updated review of *Listeria monocytogenes* in the pork meat industry and its products. *J. Appl. Microbiol*. 101, 7–17.

Tholozan, J.L., Ritz, M., Jugiau, F., Federighi, M., Tissier, J.P., 2000. Physiological effects of high hydrostatic pressure treatments on *Listeria monocytogenes* and *Salmonella typhimurium*. *J. Appl. Microbiol*. 88, 202–212.

Todd, E.C.D., Notermans, S., 2011. Surveillance of listeriosis and its causative pathogen, *Listeria monocytogenes. Food Control* 22, 1484–1490.

Todeschini, G., Friso, S., Lombardi, S., Casaril, M., Fontana, R., Corrocher, R., 1998. A Case of *Listeria murray/grayi* Bacteremia in a Patient with Advanced Hodgkin's Disease. *Eur. J. Clin. Microbiol. Infect. Dis.* 17, 808–810.

Tonello, C., 2010. *Nonthermal Processing Technologies for Food, Nonthermal Processing Technologies for Food*. Wiley-Blackwell, Oxford, UK.

Torres, J.A., Velazquez, G., 2005. Commercial opportunities and research challenges in the high pressure processing of foods*. J. Food Eng*. 67, 95–112.

Uesugi, A.R., Moraru, C.I., 2009. Reduction of *Listeria* on Ready-to-Eat Sausages after Exposure to a Combination of Pulsed Light and Nisin*. J. Food Prot*. 72, 347–353.

USDA-FSIS, 2015. Best Practices Guidance for Controlling *Listeria monocytogenes* (Lm) in Retail Delicatessens.

USDA-FSIS, 2014. FSIS Compliance Guideline: Controlling *Listeria monocytogenes* in Post-lethality Exposed Ready-to-Eat Meat and Poultry Products January 2014-143.

Vercammen, A., Vanoirbeek, K.G., Lurquin, I., Steen, L., Goemaere, O., Szczepaniak, S., Paelinck, H., Hendrickx, M.E.G., Michiels, C.W., 2011. Shelf-life extension of cooked ham model product by high hydrostatic pressure and natural preservatives. *Innov. Food Sci. Emerg. Technol*. 12, 407–415.

Waite-Cusic, J.G., Diono, B.H.S., Yousef, A.E., 2011. Screening for *Listeria monocytogenes* surrogate strains applicable to food processing by ultrahigh pressure and pulsed electric field. *J. Food Prot*. 74, 1655–61.

Wang, C.Y., Huang, H.W., Hsu, C.P., Shyu, Y.T., Yang, B.B., 2013. Inactivation and morphological damage of *Vibrio parahaemolyticus* treated with high hydrostatic pressure. *Food Control* 32, 348–353.

Warriner, K., Namvar, A., 2009. What is the hysteria with *Listeria*? *Trends Food Sci. Technol*. 20, 245–254.

Wells-Bennik, M.H.J., Karatzas, K.A.G., Moezelaar, R., Abee, T., 2008. *Listeria monocytogenes*: High hydrostatic pressure resistance and survival strategies, in: Michiels, C., Bartlett, D., Aersten, A. (Eds.), High-Pressure Microbiology. ASM Press, Washington D. C., pp. 101–116.

Wemekamp-Kamphuis, H.H., Wouters, J., Leeuw, P.P.L., De Hain, T., Chakraborty, T., Abee, T., 2004. Identification of Sigma Factor B -Controlled Genes and Their Impact on Acid Stress, High Hydrostatic Pressure, and Freeze Survival in *Listeria monocytogenes* EGD-e. *Appl. Environ. Microbiol. Env. Microbiol* 70, 3457–3466.

Werbrouck, H., Vermeulen, A., Van Coillie, E., Messens, W., Herman, L., Devlieghere, F., Uyttendaele, M., 2009. Influence of acid stress on survival, expression of virulence genes and invasion capacity into Caco-2 cells of *Listeria monocytogenes* strains of different origins. *Int. J. Food Microbiol*. 134, 140–146.

Whiting, R., Golden, M., 2002. Variation among *Escherichia coli* O157:H7 strains relative to their growth, survival, thermal inactivation, and toxin production in broth. *Int. J. Food Microbiol.* 75, 127–133.

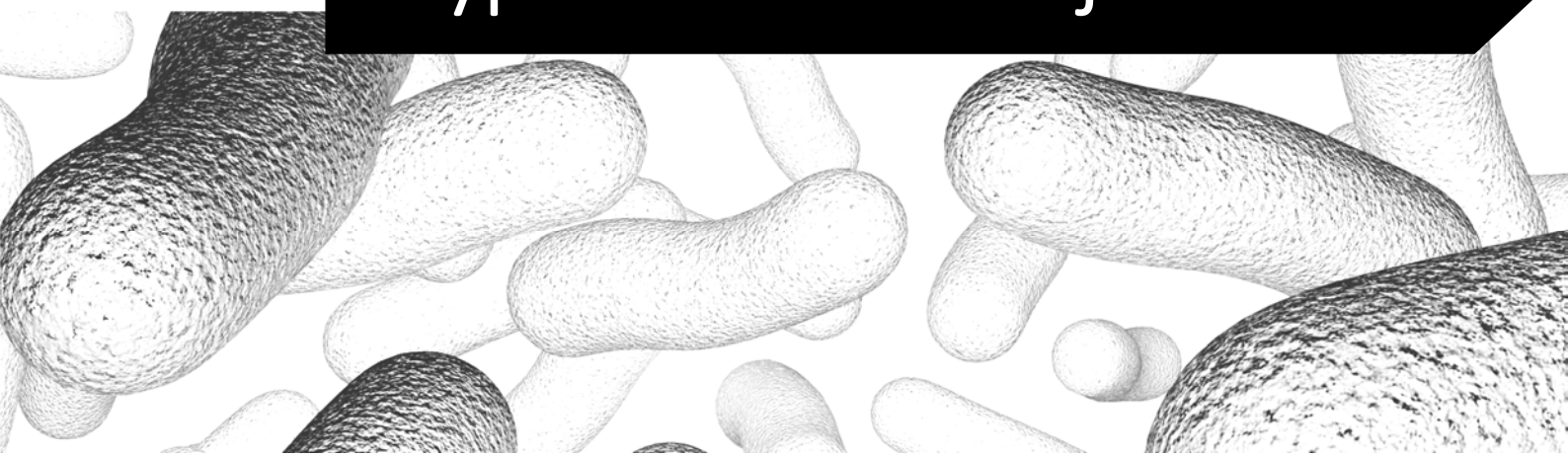
Yang, B., Shi, Y., Xia, X., Xi, M., Wang, X., Ji, B., Meng, J., 2012. Inactivation of foodborne pathogens in raw milk using high hydrostatic pressure. *Food Control* 28, 273–278.

Youart, A.M., Huang, Y., Stewart, C.M., Kalinowski, R.M., Legan, J.D., 2010. Modeling time to inactivation of *Listeria monocytogenes* in response to high pressure, sodium chloride, and sodium lactate. *J. Food Prot*. 73, 1793–1802.

Yuste, J., Capellas, M., Fung, D.Y.C., Mor-Mur, M., 2004. Inactivation and sublethal injury of foodborne pathogens by high pressure processing: Evaluation with conventional media and thin agar layer method. *Food Res. Int*. 37, 861–866.

Zhu, M., Du, M., Cordray, J., Ahn, D.U., 2005. Control of *Listeria monocytogenes* contamination in ready-to-eat meat products. *Compr. Rev. Food Sci. Food Saf*. 4, 34–42.

Hypothesis and objectives



Hypothesis and objectives

The **general hypothesis** of the study is:

Listeria monocytogenes is a foodborne pathogen, which can be found in widespread types of food and food processing surfaces. This bacterium has complex mechanisms at genetic and phenotypic level, which allow it to survive to, adapt to and grow in diverse environmental conditions. The response to stress of *Listeria monocytogenes* could vary strain to strain, although this variability depends on the type of the stress and its intensity. Hence,

"The response of *Listeria monocytogenes* to high pressure processing, a food preservation technology, depends on the strain. Moreover, this variability among strains could be influenced by both the food matrix and the processing conditions".

This hypothesis has led to the following **objectives**:

1. To evaluate the strain variability of the resistance of *Listeria monocytogenes* to high hydrostatic pressure in a buffer system and to determine correlations between pressure-resistance and the characteristics of strains.

2. To study the strain variability of the resistance of *Listeria monocytogenes* to high hydrostatic pressure in meat matrices and to determine correlations between pressure-resistance and the characteristics of strains.

3. To evaluate the suitability of (i) a cocktail of pressure-resistance *Listeria monocytogenes* strains to be used in challenge tests, and (ii) a pressure-tolerant *L. innocua* strain to be used as a surrogate of *L. monocytogenes* in RTE high pressure-processed meat products.

Hipótesis y objetivos

La **hipótesis general** del presente trabajo es:

Listeria monocytogenes es un patógeno, que puede ser encontrado en una amplia variedad de alimentos y superficies de procesamiento alimentarias. Esta bacteria tiene complejos mecanismos a nivel genético y fenotípico, lo que le permite sobrevivir, adaptarse y crecer en diversas condiciones ambientales. La respuesta al estrés de *Listeria monocytogenes* podría variar cepa a cepa, aunque dicha variabilidad depende del tipo de estrés y de la intensidad del mismo. Por tanto,

"La respuesta de *Listeria monocytogenes* frente al procesamiento por altas presiones, una tecnología de conservación alimentaria, depende de la cepa. Además, dicha variabilidad entre cepas podría verse afectada por la matriz alimentaria y las condiciones de procesamiento".

Esta hipótesis ha desencadenado los siguientes **objetivos**:

1. Evaluar la variabilidad entre cepas de la resistencia de *Listeria monocytogenes* frente a altas presiones en un buffer y determinar las correlaciones entre la resistencia a la presión y las características de tales cepas.

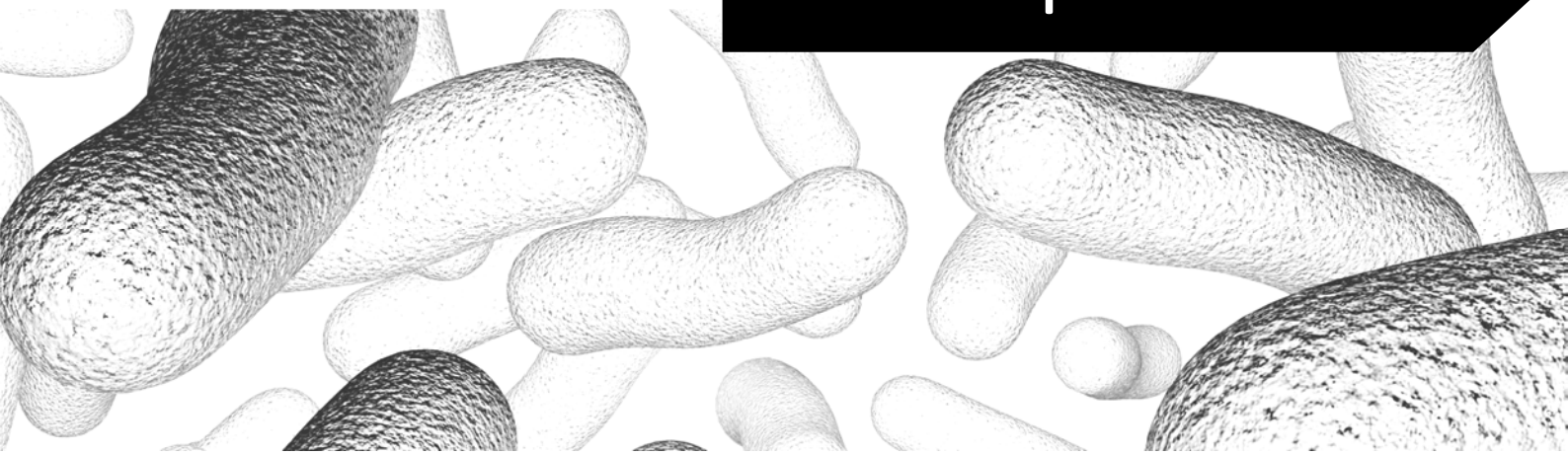
2. Evaluar la variabilidad entre cepas de la resistencia de *Listeria monocytogenes* frente a altas presiones en matrices cárnicas y determinar las correlaciones entre la resistencia a la presión y las características de las cepas.

3. Evaluar la adecuación de (i) un cóctel de cepas de *Listeria monocytogenes* resistentes a la presión para ser empleado en estudios tipo *challenge tests* y de (ii) *L. innocua* para ser usada como *surrogate* de *L. monocytogenes* en productos cárnicos listos para consumir procesados por altas presiones.

Diversity of *Listeria monocytogenes* strains response to high hydrostatic pressure.

A case study in a buffer system

Chapter 1



Chapter 1

Diversity of *Listeria monocytogenes* strains response to high hydrostatic pressure. A case study in a buffer system

Introduction

Listeria monocytogenes is a food-borne pathogenic bacteria which is ubiquitously distributed throughout the environment thanks to their ability to survive or grow in different environmental conditions, from soil and farm to food processing facilities. This allows to the pathogen to be worldwide distributed with a high prevalence (Augustin et al., 2011; Chenal-Francisque et al., 2011). Its high prevalence, morbidity and mortality compared to other foodborne illness make the control of *L. monocytogenes* a priority for health authorities. In 2013, the incidence of listeriosis was 0.44 and 0.26 cases per 100,000 population in European Union and United States, respectively. Among bacterial-foodborne diseases, listeriosis has the highest hospitalization rate (99 % in EU and 91% in US) and the highest case-fatality rate (15% in EU and 19% in US), according to European and American health authorities (Crim et al., 2014; EFSA and ECDC, 2015).

High pressure processing (HPP) is one of the available technologies for controlling effectively this pathogen in ready-to-eat products (Zhu et al., 2005). It is being commercially used by a high number of food processors in North America, Europe, Asia and Oceania. Several food safety authorities, organizations and institutions around the world recommend and accept the use of this technology for the inactivation of *Listeria monocytogenes* in different food products (FDA, 2000; FSAI, 2005; Health Canada, n.d.; USDA-FSIS, 2014).

High hydrostatic pressure can induce cell death and/or injury in vegetative microorganisms in a multitarget way, affecting cell membrane protein and fluidity, ribosome disintegration, protein and enzyme denaturation and intracellular pH shifts (Georget et al., 2015). In the specific case of *L. monocytogenes*, high hydrostatic pressure affects its morphology and physiology. Pressurized cells showed aggregated cytoplasm, occurrence of buds on cell surface and loss of membrane integrity (Mohamed et al., 2012; Ritz et al., 2001). Pressure can induce physical damage, altering the capacity of the pathogen to maintain homeostasis. The lack of control of intracellular pH, the decrease of content in intracellular potassium and ATP take place in pressurized *Listeria monocytogenes* cells (Ritz et al., 2002; Tholozan et al., 2000).

Depending on the intensity and time of high pressure treatment, these events can lead to cell death and/or injury. Although the main target of any preservation technology is to exert a lethal effect on microorganisms, the presence of injured bacteria carries on a hazard in food production (Bozoglu et al., 2004). The presence of injured bacteria is risky for two reasons. Firstly, their sensitivity to agents to which would otherwise show resistance hinders its growth in conventional selective media, increasing the risk of false-negative results. Secondly, the injured microorganisms are able to recover if the environmental conditions are favorable (Abe, 2007; Jasson et al., 2007). The physicochemical characteristics of food, its composition and storage conditions will determine if the injured microorganisms will be able to recover and grow, or otherwise die during storage. Thus, the quantification of this state for any preservation technology, including HPP, is relevant to microbial risk assessment approach.

Although the high hydrostatic pressure technology has demonstrated to be effective to control pathogenic and spoilage microorganisms, their response to pressure can vary at species level. In general, protozoans, molds and yeasts are more sensitive to high pressure than bacteria and viruses (Abe, 2007). Among bacteria, Gram-positive, stationary phase and spore state seemed to be more pressure-tolerant than Gram-negative, exponential growth phase and vegetative cells. Even, differences strain-to-strain within a same species have been reported (Alpas et al., 2000).

Food microbiologists often assume that strain variability of microbial behavior is equal to or smaller than the experimental variation. However, strain selection is a critical decision when designing and conducting challenge tests (Lianou and Koutsoumanis, 2013). Strains with above average, but not with unrealistic extreme resistance, or those associate with public health concerns are preferred to be included in challenge testing (Balasubramaniam et al., 2004), because their approximation of the results to a real case scenario.

L. monocytogenes showed a strain-dependent resistance to different preservation technologies such as bacteriocins (Rasch and Knøchel 1998); pulsed electric fields (Lado and Yousef, 2003) and thermal treatment (Aryani et al., 2015; Doyle et al., 2001). Although some studies determined the variability of pressure-resistance of the pathogen (Alpas et al., 1999; Simpson and Gilmour, 1997a; Tay et al., 2003), the results are limited due to the low number of strains evaluated, usually less than ten, which is insufficient to cover the wide diversity within this species.

The diverse geno- and phenotypic characteristics of *L. monocytogenes* give to the subtypes of the species the ability to withstand with different stress conditions, exerting varied responses to grow and express virulence (Arguedas-Villa et al., 2010; Barmpalia-Davis et al., 2008). Subtypes can be classified in different ways, being lineage, serogrouping, and virulence-related factors the most commonly used. The species diversity can be represented in four lineages based on differences in listerial genome, apparently caused by recombination events. These lineages represent diverse ecologic, genetic and phenotypic characteristics related to the ability to be transmitted through foods and to cause human disease (Orsi et al., 2011). For instance, lineage I, the lowest diverse among lineages, contains the most of strains related to listeriosis-outbreak cases. In contrast, isolates from food, food processing and natural environments are overrepresented in lineage II, the most diverse. Lineages III and IV are comprised by strains isolated from animals, mainly ruminants (Abu Mraheil, 2013; Orsi et al., 2011). According to their reactivity against specific antibodies, *L. monocytogenes* is classified in 13 serotypes. Its importance is related to its correlation with pathogenicity (Bourdichon, 2014; Nadon et al., 2001), being serotypes 1/2a and 4b the most common isolates in

human cases (> 90 %) in Europe (EFSA and ECDC, 2015). Routine analysis of *Listeria monocytogenes* by serotyping with traditional agglutination methods is limited by cost, availability, and the need for technical expertise to perform the assay. Thus, a method for PCR-based serogrouping is proposed as an alternative for cheap, fast and reliable results (Doumith et al., 2004). The method classifies strains in four serogroups and it is currently used by some European countries. The reports showed that the most common isolates from clinical cases belonged to serogroup 2 and 4 (EFSA and ECDC, 2015). Genetic diversity has been shown among human isolates characterized by unique virulence or host specificity patterns. However, more research is needed to differentiate the highly virulent from the less virulent strains of *L. monocytogenes* (Luber et al., 2011). On this regard, Liu et al. (2007b) developed a multiple PCR-based method to detect the presence of three internalin genes (*inlA, inlC* and *inlJ*), which are highly correlated to *in vitro* virulence. Internalins are surface proteins used by the pathogen for entering into eukaryotic cells during invasion step. Those strains in which internalins genes were absent, were significantly less pathogenic (Liu et al., 2007a), therefore they have been proposed as virulence factor markers.

Some strains of *L. monocytogenes* have exhibited the presence of high pressure-resistant subpopulations, which have been characterized geno- and phenotypically (Karatzas et al., 2007; Liu et al., 2011; Van Boeijen et al., 2010). Those studies have helped to understand the stress response mechanisms at genetic level, elucidating partly how the pathogen is able to withstand hydrostatic pressure. Despite the previous information about pressure-resistance strain variability, the low number of wild-type strains and the behavior of those mutants is not sufficient for covering the natural diversity of this pathogenic species.

The objective of this study was to evaluate the variability of the response (inactivation and sublethal injury) to high hydrostatic pressure of strains from three *L. monocytogenes* collections from USA and Spain. The strains were characterized geno- and phenotypically, in those cases which there were no previous details. Information about lineage, serogroup, origin, virulence factors and listeriosis-case history was

reported in order to determine correlations between those characteristics and pressure-resistance.

Materials and methods

Strain selection

One-hundred and twenty *L. monocytogenes* strains were characterized in this study. They came from three collections: Forty four, 55 and 21 strains from International Life Science Institute – North America (ILSI, USA), Technical Agriculture Institute of Castilla y León (ITACyL, Spain) and University of Burgos (UBU, Spain) were included, respectively. As a preliminary step, PCR serogrouping, internalins presence and a biochemical characterization was done according to the methodology described below. A *L. innocua* strain from UBU collection was included in this study, as well.

Culture preparation

The stock cultures were kept frozen at −80 °C in 70 % brain heart infusion (BHI) broth (Oxoid, UK) and 30 % glycerol (v/v) (Sigma Aldrich, Spain). From the stock culture, a streak of each strain was made onto a tryptone soy agar and 0.6 % yeast extract (TSAYE, Oxoid, UK) plate and incubated for 24 h at 37 °C. A single colony was inoculated in a test tube pre-filled with 5 mL of BHI broth and incubated until the stationary phase for 18 h at 37 °C. Serial dilutions were done until reaching 10^7 colony-forming units (CFU)/mL by 9 mL-test tubes pre-filled with phosphate buffer solution (PBS, Oxoid, UK). Four milliliters of each inoculum were transferred aseptically to a sterile plastic Pasteur pipette, then were heat-sealed, vacuum-packaged in plastic bags and immediately stored at 4 °C before HPP treatments (< 2 h).

DNA extraction

The DNA used for PCR assays was extracted from 2 mL of cell culture using the GenElute™ Kit (Bacterial Genomic DNA Kit, Sigma, USA) according to the manufacturer's recommendations for Gram-positive bacteria. DNA obtained was resuspended in 100 µL

of the recommended buffer. The extracted DNA was quantified using an Epoch Bioteck spectrophotometer and the GEN 5 v2.00 software (Biotek, Vermont, USA).

PCR serogrouping and presence of internalins genes

Serogrouping of the *L. monocytogenes* strains were done following the protocol of Doumith et al. (2004) with some variations. A multiplex PCR amplifications were performed in a final sample volume of 25 µL containing 1× PCR reaction buffer without $MgCl_2$ (Sigma), 200 µM of each dNTP (dATP, dCTP, dGTP, dUTP) (Promega, Madison, WI), 1 µM of *lmo0737*, ORF2110 and ORF2819; 1.5 µM of *lmo1118* and 0.2 µM of *prs* primer (Sigma), 2 mM of $MgCl_2$ (Sigma), 0.5 U of *Taq* DNA Polymerase (Sigma) and 100 ng of template DNA.

Internalins genes, *inlA, inlC* and *inlJ*, were detected in each strain according to the method designed by Liu et al. (2007b). The multiplex PCR was conducted in a volume of 25 µL containing 0.8 U *Taq* DNA polymerase 1× PCR buffer, 200 µM dNTPs and 10 ng each *Listeria monocytogenes* DNA, together with 40 pmol each *inlA*, 30 pmol each *inlC* and 20 pmol each *inlJ* primers. All material was provided by Sigma Aldrich (Spain).

All PCR reactions were performed using a Mastercycler gradient thermal cycler (Eppendorf, Spain). The PCR products were separated by electrophoresis on a 2.0 % Electran agarose gel (VWR, UK) and stained with ethidium bromide (Amresco, USA).

Biochemical characterization

Strains were further subjected to haemolysis on Christie, Atkins, Munch Petersen (CAMP) test on Columbia blood agar (Oxoid, UK) and detection of phosphatidylinositol-specific phospholipase C (PI-PLC) activity on agar *Listeria* according to Ottaviani and Agosti (ALOA) medium (Oxoid, UK).

High pressure processing conditions

Five conditions of pressure and holding time were tested: 400 MPa, 3 s; 400 MPa, 360 s; 500 MPa, 180 s; 600 MPa, 3 s and 600 MPa, 360 s at room temperature (20 °C) in a 135-L high pressure unit (Hiperbaric 135 from Hiperbaric S. A., Burgos, Spain), using water as pressurization liquid. The pressure build-up time was 130, 150 and 170 s for

400, 500 and 600 MPa, respectively. Depressurization time was less than 3 s. The range of pressure and holding time was selected to cover usual industrial conditions.

Determination of inactivation and sublethal injury

Differential plate counting was performed before and after high pressure processing in TSAYE for enumeration of viable cells, and in TSAYE with NaCl (40 g/L) for enumeration of no-injured cells only, according the recommendations of Jasson et al. (2007) and Jofré et al. (2010). The number of inactivated cells was estimated by the difference between counts in TSAYE of control and pressure-treated sample, whereas the quantification of sublethally injured was done by the difference of counts between TSAYE and TSAYE with NaCl. The results were expressed in $\log_{10}$ CFU/mL.

Data analysis

All strains were analyzed in triplicate for each HPP treatment. Cluster analysis on the complete dataset was performed by a euclidic distance analysis using the Ward method on the non-scaled data (OriginPro 9.0, Originlab, USA). Statistically significant differences between clusters according to the characteristics of the strains (lineage, serogroup, virulence, pathogenicity or the source of isolation) were identified by analysis of variance (Statgraphics Centurion XVI, StatPoint Technologies, Inc., USA). All statistical procedures were done on inactivation and sublethal injury data expressed in $\log_{10}$ CFU/mL.

Results and Discussion

Strain selection

The 120 *L. monocytogenes* strains presented a wide diversity according their geno- and phenotypical characterization (**Figure 1**). Strains from the four serogroups were determined. Around one third of strains belonged to serogroup 4 (serotypes 4b, 4d and 4e), followed by serogroup 1 (serovars 1/2a and 3a) with 25 % of strains. Serogroup 2 (serovars 1/2c and 3c) and serogroup 3 (1/2b, 3b and 7) represented the 26 % of the strain collection. The remaining 21 % of strains had a serogroup profile not covered, thus

they were classify as "*Listeria* spp." group, according to the original method by Doumith et al. (2004). Lineage of those strains with no previous details were determined using the correlations with serotype given by Nadon et al. (2001). Lineage I (49 %) were the largest phylogenetic group, followed by lineage II, representing together around 87 % of the strains. The remaining 13 % consisted in strains from lineages III and IV. The strain collection covered the four evolutionary lineage of the pathogen.

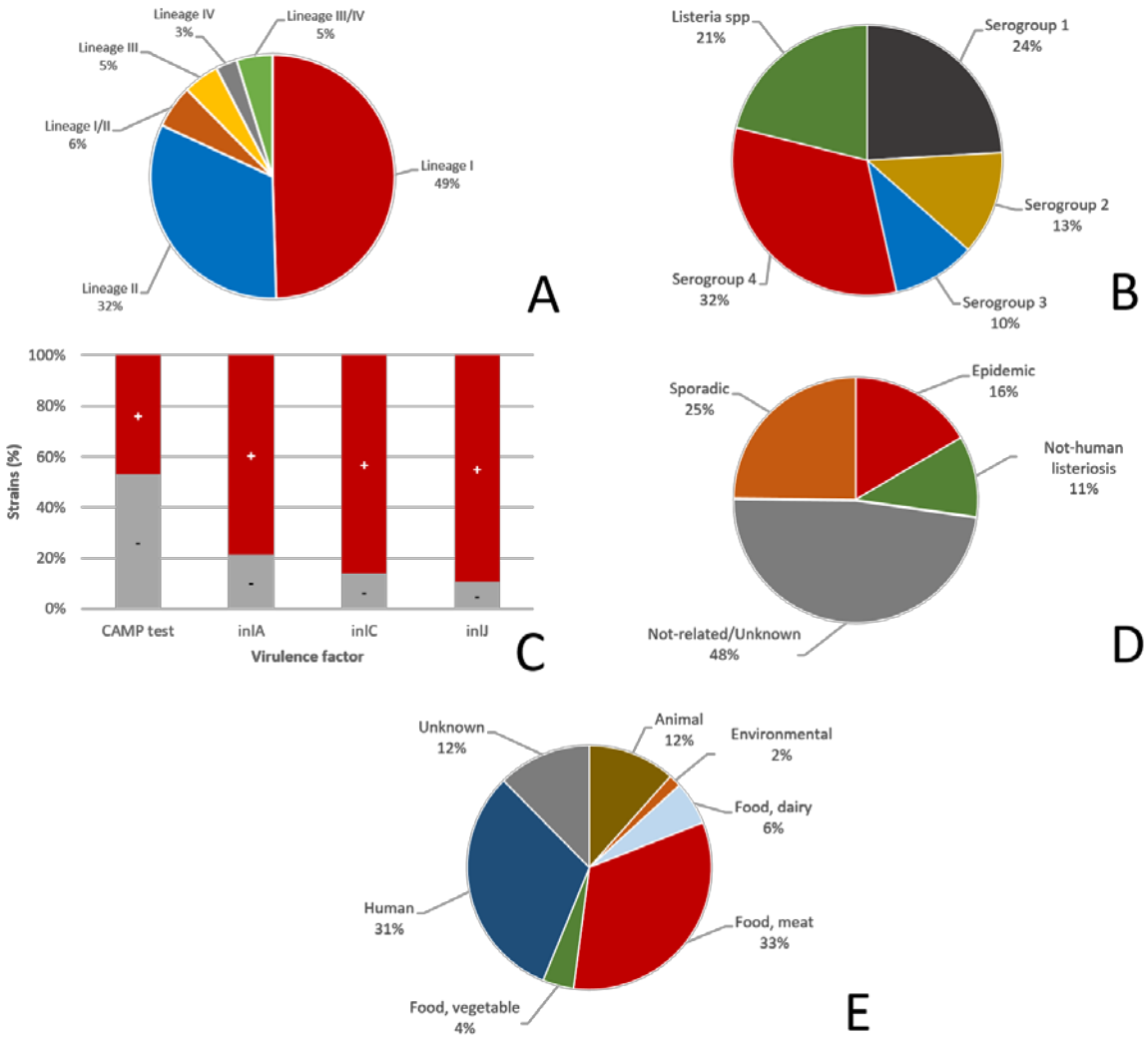


Figure 1. Characterization of the 120 *L. monocytogenes* strains according their source of lineage (A), PCR serogrouping (B), virulence factors (C), listeriosis-case history (D) and isolation (E)

All the *L. monocytogenes* strains exhibited PI-PLC activity, whereas the half exhibited a clear haemolytic activity according to the CAMP test. Ninety-two strains had the three internalin genes (*inlA, inlC* and inlJ) present. According to our results, more than a half of strains can be classified as potentially virulent. In addition, the 16 % have been associated to listeriosis outbreak, 25 % to sporadic human listeriosis cases, and 11% associated to listeriosis in animals. Less than a half of the strains had unknown listeriosis-cases associated history. Fifty-five strains were isolated from food products (44 %), coming from meat (n = 40; 33 %), dairy (n = 7; 6 %) and vegetable products (n = 5; 4%). Strains from human clinical cases represented the 31%, 12 % from animal clinical cases and 2 % from environmental samples. Fifteen strains (12 %) were isolated from unknown origin.

Based on this information and previous details given by the collection owners, we selected 30 strains for high pressure treatment trials (**Table 1**). These strains were selected from the initial 120, seeking to cover a wide range of lineage, serogroup, the source of isolation, virulence factors and known pathogenicity, eliminating those strains with a similar profile.

Regarding the selected set of strains, lineage I (n = 15) was the most represented, followed by Lineage II (n = 10). Four strains from Lineage III and IV and two strains with no lineage information were included, as well. The selection of most of the strains from Lineage I and II is reasonable because their direct relationship with human clinical cases and their persistence in food and food processing environments, respectively (Orsi et al., 2011). Nevertheless, strains from all serogroups were included. Seven strains from serogroup 1, four from serogroup 2, five from serogroup 3 and nine from serogroup 4 (4b, 4d and 4e) were included. Six strains classified as "*Listeria* spp." serogroup were included, as well. Five of them were identified as *L. monocytogenes.* The sixth strain corresponded to *L. innocua* UBU.

Regarding the virulence-associated factors, around half of the strains showed significant haemolytic activity. Five out of the 30 strains were positive of the three internalins genes (*inlA*, *inlC* and *inlJ*), potential virulence markers (Liu et al., 2007b). The selected set comprised epidemic- (n = 9) and sporadic-related strains (n = 14). The rest of strains (n

= 8) have not been related to clinical cases. Animal (n = 4) and human (n= 12) isolates were included. The selected set comprised twelve isolates from food products (five from dairy, four from meat and three from vegetable products). Three strains from unknown origin were included, as well.

High hydrostatic pressure induces diverse inactivation levels depending on the strain

The distributions of inactivation levels caused by high hydrostatic pressure on *Listeria monocytogenes* strains depended on the applied pressure and holding time (**Figure 2**). At the lowest (400 MPa, 3 s) and the highest (600 MPa, 360 s) conditions, the inactivation variability was lower than the rest of tested conditions. Low inactivation was achieved for the most of the strains (< 1 log CFU/mL) at 400 MPa for 3 s. Processing at 600 MPa for 360 min achieved above 5 log-inactivation in all strains. For intermediate treatments, the inactivation range were wider. At 400 MPa for 360 s, most of the strains presented an inactivation value below 2 log-cycles. However, a group of more sensitive strains were inactivated in a range from 2 to 4 log CFU/mL. Similarly, at 500 MPa for 180 s, there were two groups of strains.

Most of the strains exhibited a low tolerance at these conditions, achieving from 3.0 to 6.5 log-inactivation. Few strains, the most resistant, were inactivated less than 2 log cycles. Six-hundred megapascals during few seconds exerted a widespread inactivation in the *L. monocytogenes* strains, from no-inactivation up to 5.5 log CFU/mL. These broad ranges of inactivation are in agreement with previous works about variability of pressure tolerance of different strains of this foodborne pathogen, however, it has not been described at different HPP conditions in a same set of strains (Alpas et al., 1999; Chen et al., 2009; Simpson and Gilmour, 1997b; Tay et al., 2003; Youart et al., 2010). Chen et al. (2009) found reductions between 1.9 to 7.1 log CFU/mL at 400 MPa for 2 min at 21 °C of 30 strains in tryptic soy broth, while Youart et al. (2010) found a 5-log difference between pressure-sensitive and resistant strains at 600 MPa for 60 s.

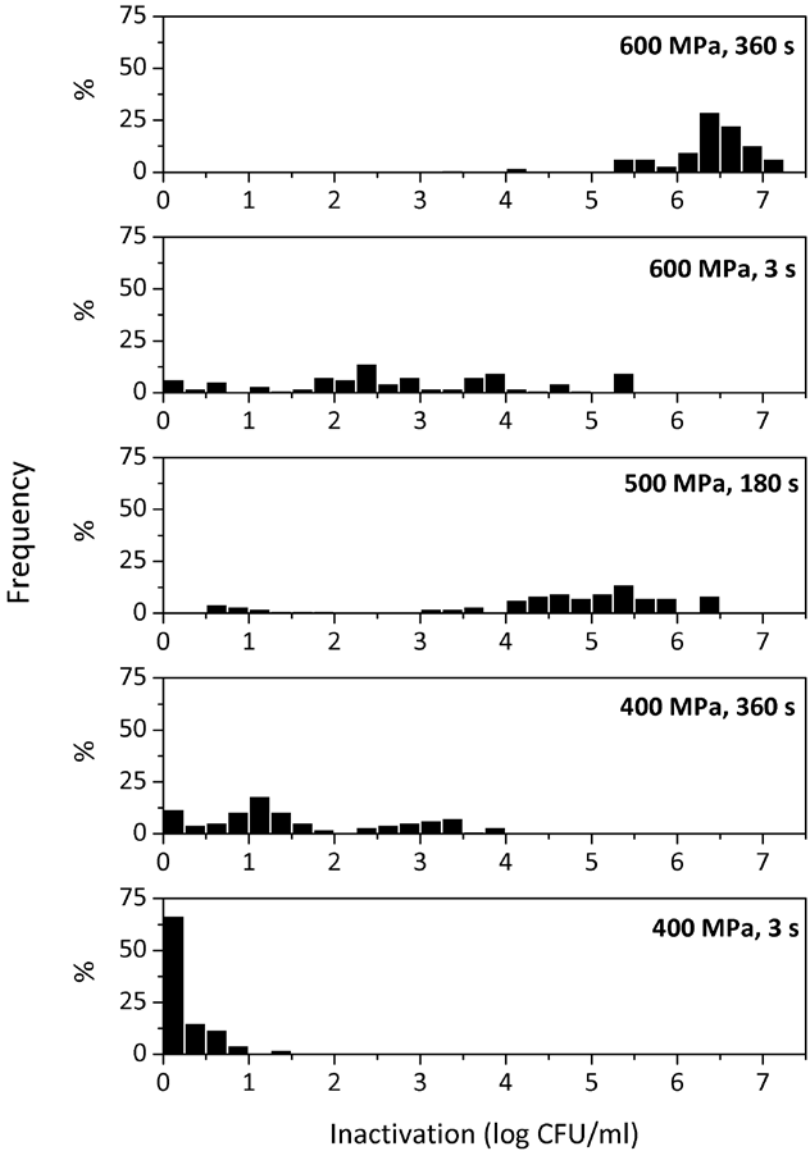


Figure 2. Distribution of inactivation (log CFU/mL) of the 30 *Listeria monocytogenes* strains in PBS buffer by high hydrostatic pressure at 600 MPa, 360 s; 600 MPa, 3 s; 500 MPa, 180 s; 400 MPa, 360 s; and 400 MPa, 3 s.

The variable response at strain level to high hydrostatic pressure has been observed in different microorganisms other than *L. monocytogenes* such as *E. coli*, *Campylobacter* spp., *S. aureus,* and Hepatitis A virus (Alpas et al., 1999; Benito et al., 1999; Gänzle and Liu, 2015; Garcia-Hernandez et al., 2015; Shimasaki et al., 2009). Besides pressure, *Listeria monocytogenes* exhibits strain-dependent response to other preservation technologies such as thermal treatment (Aryani et al., 2015; Doyle et al., 2001),

bacteriocins (Rasch and Knøchel, 1998), and pulsed electric fields (Lado and Yousef, 2003).

Sublethal injury caused by high pressure varies strain to strain

High pressure processing caused different sublethal injury levels on *L. monocytogenes*, depending on the strain, pressure and holding time (**Figure 3**). Analogous to inactivation, at the lowest and highest intense conditions (400 MPa, 3s and 600 MPa, 360 s), the injured values were below 1 log CFU/mL for most of the strains. Although both treatments exerted similar results, the causes were different. The counts of dead and injured *L. monocytogenes* cells were low at 400 MPa for 3 s, whereas at 600 MPa for 360 s, processing led to values close to total inactivation, explaining the low counts of survivors, including the injured ones.

The higher variability was found at intermediate pressure-time conditions (400 MPa for 360 s, 500 MPa for 180 s and 600 MPa, 3 s). Most of the strains presented between 4 to 6 log-injury at 400 MPa for 360 s, indicating that at these conditions it was possible to provoke sublethal injury in the survivors of pressure-treated *L. monocytogenes.* Most of strains were injured less than 3 log CFU/mL at 500 MPa for 180 s. Similarly to inactivation, pressure-treated *L. monocytogenes* exhibited a widespread sublethal injury levels after 600 MPa during 3 s. Although this holding time is below industrial conditions, the latter result pointed out the relevance of holding time as processing parameter. At the maximum pressure at industrial level, longer holding times permitted to reach more than 5-log inactivation of the pathogen with a low quantity of injured cells, whereas a extremely short times, the lethal and sublethal effect will be highly dependent of the strain. The variability in high hydrostatic pressure resistance of the different species, strains and even cells within a population makes the proper design of HPP treatments that would allow adequate reductions of bacteria a challenging task (Wells-Bennik et al., 2008).

Few studies have been done about the assessment of strain variability in pressure-injured bacteria. Alpas et al. (2000) found differences in the percentage of injury in two

strains of *L. monocytogenes* and *S. aureus* at pressures up to 345 MPa for 5 and 10 min at different temperatures. *L. monocytogenes and Salmonella enterica* did not show significant strain-specific differences in cell injury after high pressure treatment at 400, 600 and 900 MPa, while higher variability were detected in *S. aureus* strains (Jofré et al., 2010).

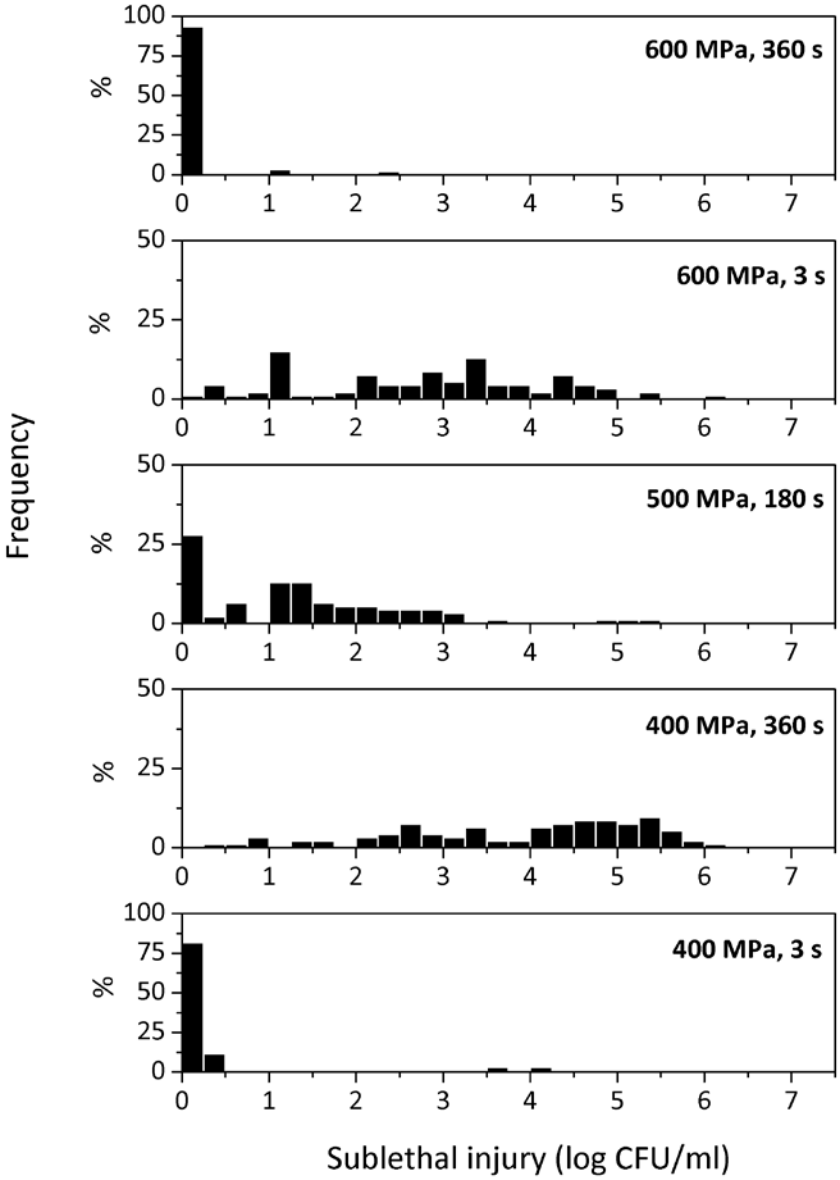


Figure 3. Distribution of sublethal injury (log CFU/mL) of the 30 *Listeria monocytogenes* strains in PBS buffer by high hydrostatic pressure at 600 MPa, 360 s; 600 MPa, 3 s; 500 MPa, 180 s; 400 MPa, 360 s; and 400 MPa, 3 s.

L. monocytogenes strains group in different clusters according their pressure-resistance

Since the lethal and sublethal effect of high hydrostatic pressure on strains depended on pressure and time, a cluster analysis was done for grouping those strains which behave similarly. This type of multivariate analysis has been previously done for classifying strains according their ability to handle environmental conditions, but never for high hydrostatic pressure (Begot et al., 1997; Van Boeijen et al., 2010; Van Der Veen et al., 2008). The results of this cluster analysis, for inactivation and sublethal injury data, are reflected in the dendrograms (**Figure 4** and **Figure 6**, respectively).

According to their ability to resist to high hydrostatic pressure, the thirty strains of *L. monocytogenes* and the strain of *L. innocua* were grouped in three clusters for inactivation data (**Figure 4**). The three groups (A, B, C) had an heteregeneous number of strains, being cluster A (n = 18) the largest group, followed by B (n = 9) and C (n= 4). Cluster C was the most resistant group with a mean of less than 1 log-inactivation for all HPP conditions except at 600 MPa for 360 s (6.3 ± 0.5 log-inactivation). Cluster A presented a similar pattern of inactivation to cluster B ($p > 0.05$), around 5 and 3 log-inactivation at 500 MPa for 180 s and 600 MPa for 3 s, respectively. However, at 400 MPa for 360 s, cluster A (1.1 ± 0.5 log CFU/mL) exhibited a higher pressure-tolerance than cluster B (3.1 ± 0.4 log CFU/mL).

Pressure and holding time affected in different way each cluster (**Figure 5**). Each processing parameter affected significantly ($p < 0.001$) the inactivation levels of all the clusters. The interaction of these parameters affected significantly ($p < 0.001$) the inactivation levels in clusters A and C. However, pressure and holding time affected inactivation of Cluster B in a additive way ($p_{interaction} > 0.05$). Our results suggested the pressure-resistance of clusters was C > A > B.

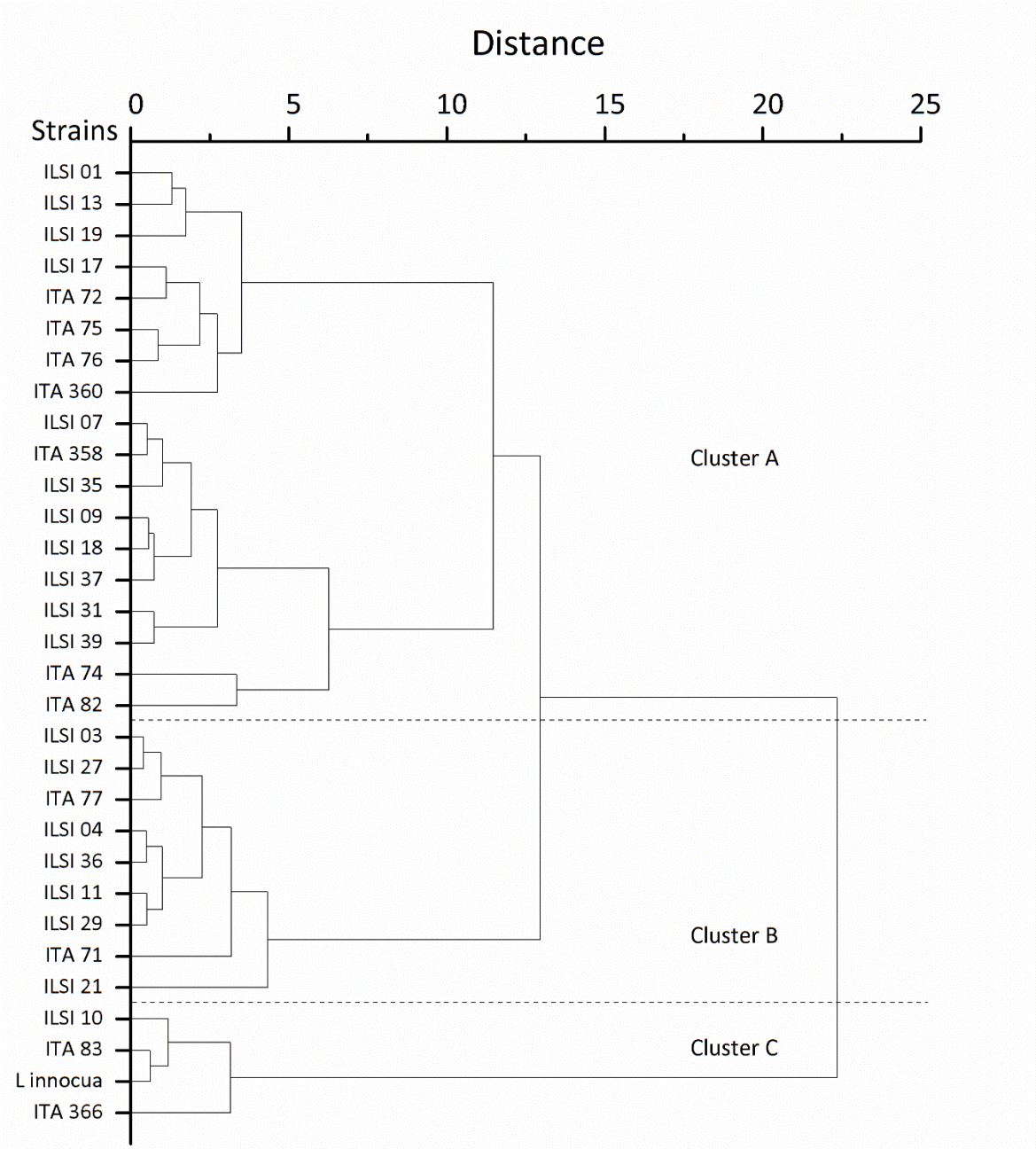


Figure 4. Cluster dendrogram of the inactivation of 30 *Listeria monocytogenes* strains and one *L. innocua* by high hydrostatic pressure. The strains were clustered by using euclidic distance and Ward grouping method.

Strains were classified in three major groups (X, Y and Z) according the injury levels caused by high hydrostatic pressure (**Figure 6**). The three clusters had a similar number of members (10, 12 and 9 for cluster X, Y and Z, respectively) and there was no correspondence between the resulting clusters from inactivation and sublethal injury data. Such as the distributions showed (**Figure 3**), treatments at 400 MPa for 3s and 600 MPa for 360 s exerted low injured values (< 1 log CFU/mL). Hence, the variability of the clusters X, Y and Z lied on the intermediate conditions. Strains contained in cluster Z were the most susceptible to be injured by high hydrostatic pressure ($p < 0.05$) at the three intermediate conditions, whereas cluster Y presented the lowest values of sublethal injury (up to 2.6 log CFU/mL). Cluster X had a similar injury pattern to cluster Y, however, processing at 400 MPa for 360 s caused higher injury in cluter X than cluster Y (4.5 ± 1.2 log CFU/mL and 2.6 ± 1.1 log CFU/mL, respectively).

Similarly to inactivation, pressure and holding time caused cell injury to each cluster in a different levels. Analysis of variance showed pressure-time interaction affected significantly ($p < 0.001$) the sublethal injury of all clusters. Interaction between processing parameters is evident in the contour plots (**Figure 7**). At 400 MPa, strains were more susceptible to be injured at longer holding times. On the contrary, higher injured levels were caused at 600 MPa during 3 s. According to our results, cluster Z was the most susceptible to be pressure-injured, followed by X and Y.

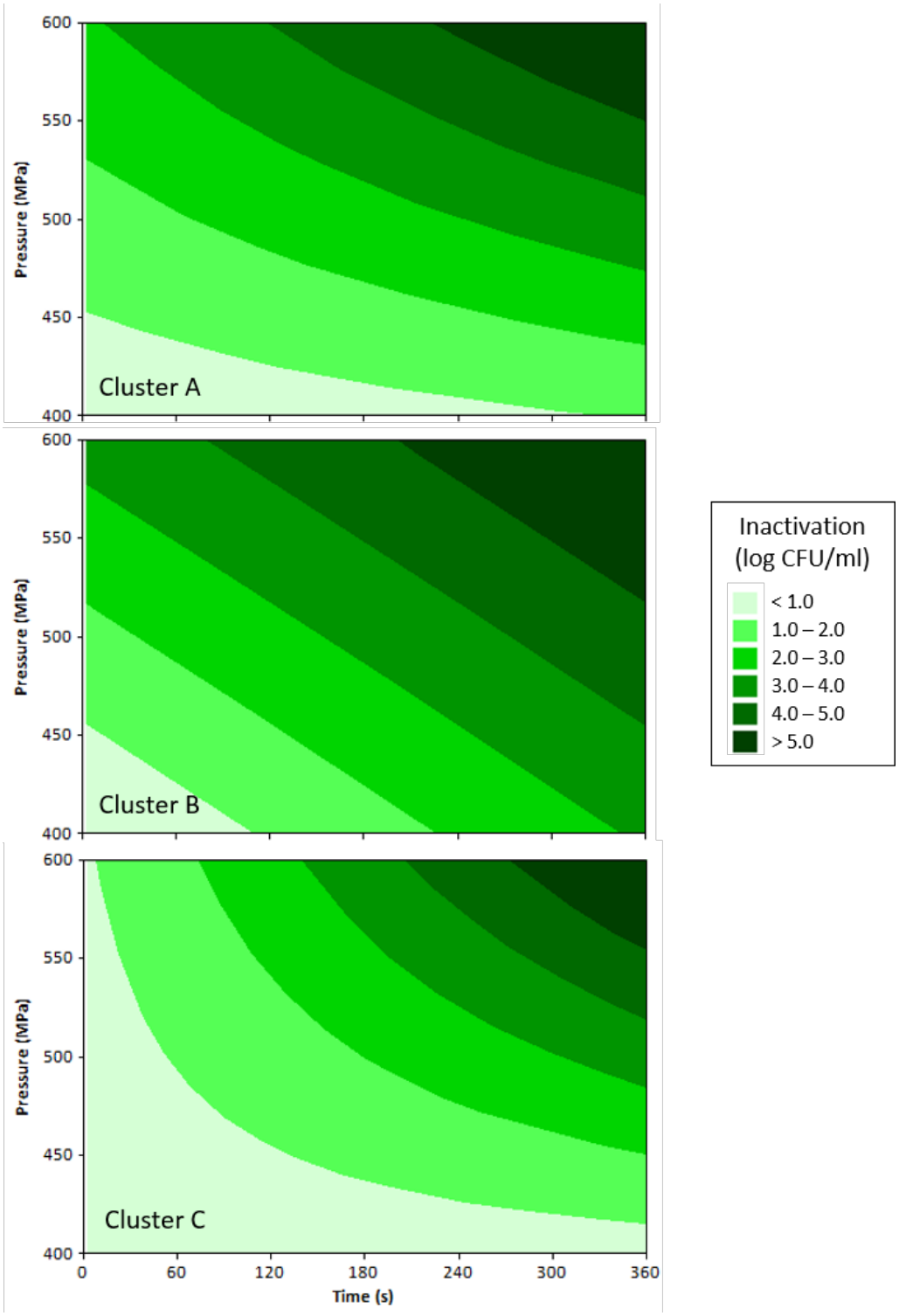


Figure 5. Contour plots of inactivation levels of *Listeria monocytogenes* clusters

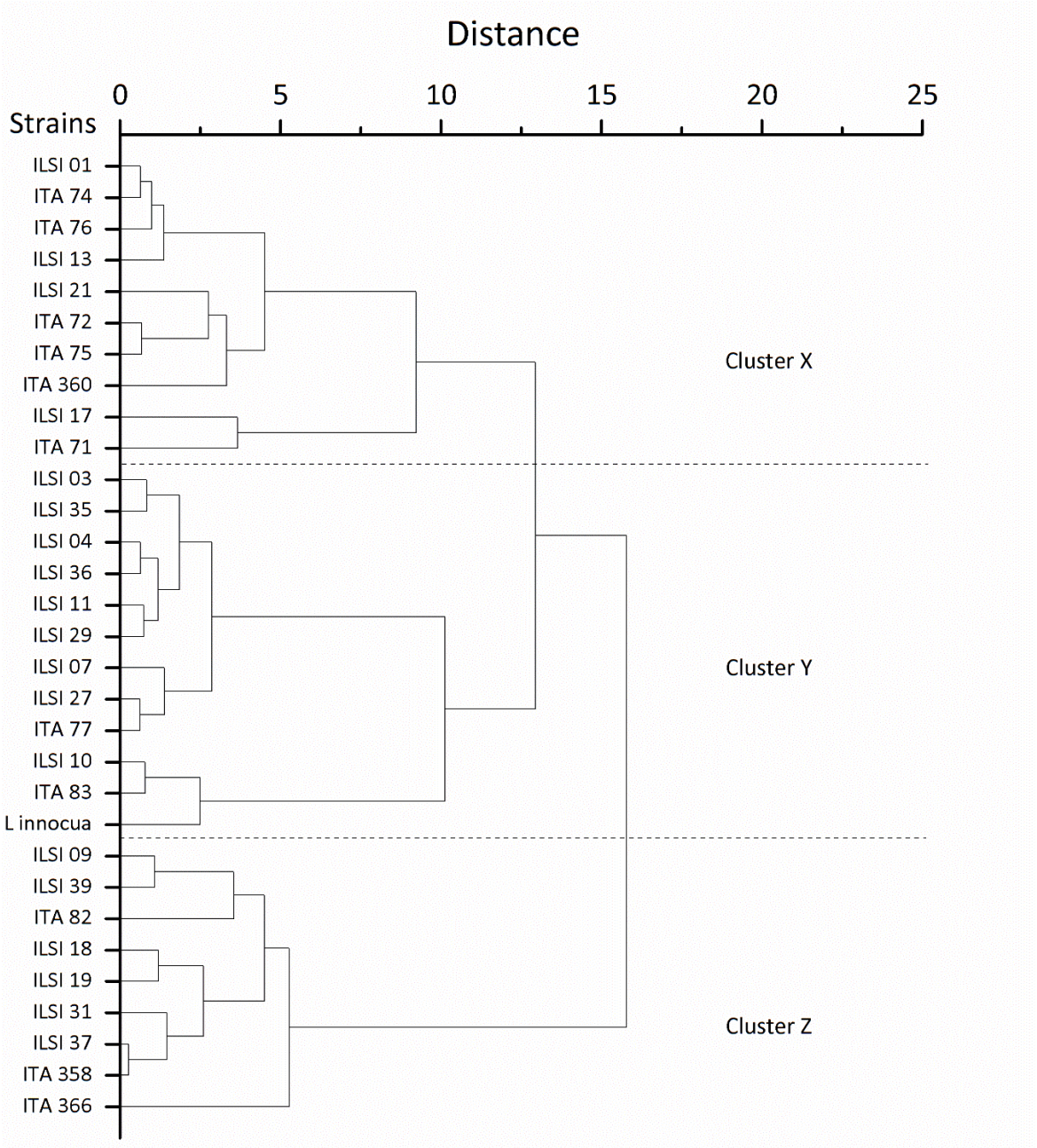


Figure 6. Cluster dendrogram of the sublethal injury of 30 *Listeria monocytogenes* strains and one *L. innocua* by high hydrostatic pressure. The strains were clustered by using euclidic distance and Ward grouping method.

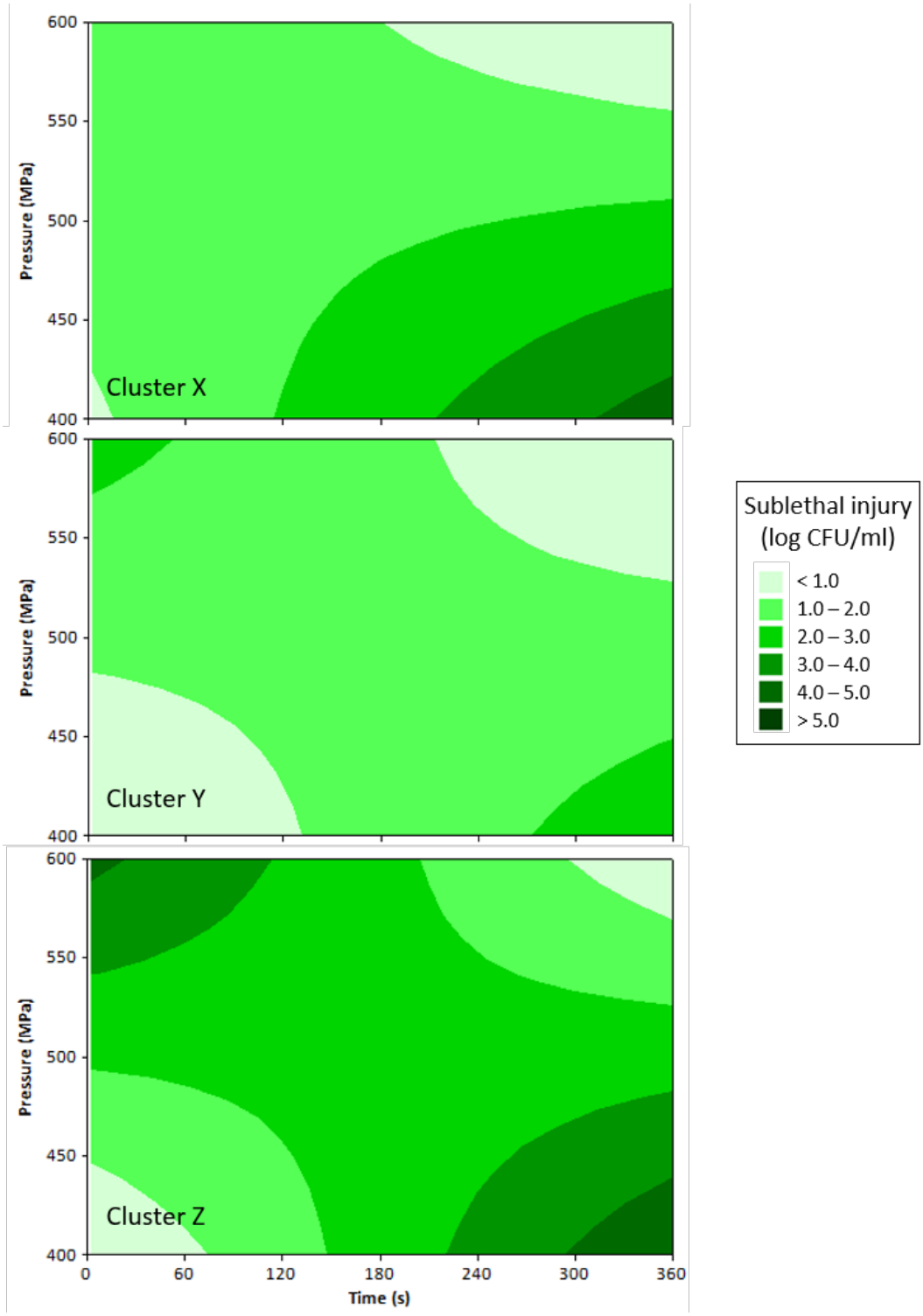


Figure 7. Contour plots of sublethal injury levels of *Listeria monocytogenes* clusters

Potential reference strains for using in high hydrostatic pressure challenge studies

Members of cluster C (ILSI 10, ITA 83 and ITA 366) were the most pressure-resistant (**Table 1**) and they can be potential strains for using in studies about the control of *L. monocytogenes* by high hydrostatic pressure. None of these strains has been associated to outbreaks cases, however, ILSI 10 has pathogenic behavior, being isolated from a sporadic human clinical case and ITA 83 and ITA 366 presented the virulence-associated factors evaluated in this work. Besides, the three strains belong to a different serogroup, faciliting tracing them by using the PCR serogrouping method. These three strains could be included in a five-strains cocktail for use in validation or challenge studies as it is recommended (Balasubramaniam et al., 2004; NACMCF, 2005).

Among *L. monocytogenes* strains, Scott A, a milk-borne isolate with clinical importance, is often used as a reference strain, also in studies about high hydrostatic pressure (Simpson and Gilmour, 1997b; Tay et al., 2003; Van Boeijen et al., 2008; Waite-Cusic et al., 2011). We tested the pressure-resistance of this strain (coded as ILSI 01). The strain was grouped in cluster A and cluster X, showing an intermediate-low resistance to high hydrostatic pressure, in agreement with other authors (Simpson and Gilmour, 1997b; Tay et al., 2003; Van Boeijen et al., 2008), questioning its suitability as reference strain for HPP validation studies. Based on the results from this study, we propose the use of other strains for processing validation purposes and research such as ILSI 10, ITA 83 and ITA 366 which demonstrated higher resistance to high hydrostatic pressure than Scott A strain.

The strain of *Listeria innocua* belonged to clusters C and Y. The members of these clusters exhibited a high resistance to pressure-induced death and injury. This species of *Listeria* genus is usually used as surrogate of *L. monocytogenes* in challenge testing of different preservation technologies due to its similar behavior, easy-handling and its lack of pathogenicity. In the case of high hydrostatic pressure, Santillana-Farakos and Zwietering (2011) found no significant differences in log D_p values between both species of *Listeria* genus. However, other authors stated its pressure-resistance is intermediate

compared to other *L. monocytogenes* strains and other potential surrogates (Tay et al., 2003; Waite-Cusic et al., 2011). Our results suggested *L. innocua* UBU strain is a potential surrogate for validation of high pressure processes, due to its high tolerance and similar susceptibility to be pressure-injured compared to 30 *L. monocytogenes* strains, its lack of pathogenicity, reproducible growth, easily enumerated, characteristics reccommended for surrogate organisms (NACMCF, 2010). Further studies should be done to validate the use of this microorganism as surrogate in real food products.

Correlation between pressure-resistance and the characteristics of the strains

The characteristics of the evaluated strains and their cluster membership are summarized in **Table 1**. Based on these results, there was no a clear correlation between clusters and lineage, serogroup, haemolysis activity, internalins gene presence and listeriosis history ($p > 0.05$). Most of lineage I strains (10 out of 15), which are related with most of cases of human clinical isolates (Orsi et al., 2011), were found in Cluster A. Despite the fact that cluster A appearently contained the most of human cases isolates, there was no a correlation between cluster and lineage ($p > 0.05$). Serogroup 4, which contains serotype 4b (the most related to listeriosis cases) was present in cluster A as well as in cluster B. The same two clusters contained the most of epidemic- and sporadic-related strains. Liu et al. (2015) found no correlation between pressure resistance and phylogenetic group or serotype of a representative set of *E. coli* strains. In the case of *Listeria monocytogenes*, the correlation between resistance and phylogenetic or serotype depends on the stress which is been subjected. Salt-stress (Bergholz et al., 2010; Van Der Veen et al., 2008), biofilm formation (Kadam et al., 2013) and survival to simulated gastrointestinal tract model (Barmpalia-Davis et al., 2008) were strongly correlated to serotype and phylogenetic characteristics. By contrast, the tolerance to cold stress (Arguedas-Villa et al., 2010), bacteriocins (Rasch and Knøchel, 1998), pulsed electric fields (Lado and Yousef, 2003) and thermal treatment (Francis and O'Beirne, 2005) were not related to phylogenetic or serotype differences among the strains tested.

The latter reflects the complexity of resistance mechanisms in a microorganism such as *L. monocytogenes.* Although the pressure-resistance phenomena is not completely elucidated, some studies have demonstrated the role of genetic factors such as sigma factor σ^B-dependent genes (Wemekamp-Kamphuis et al., 2004). Sigma factor σ^B plays a central role in redirecting gene expression under stress conditions, and its activation confers protection to a wide range of stress in *L. monocytogenes* and other Gram-positive bacteria (Chaturongakul et al., 2008; Wells-Bennik et al., 2008). Besides Sigma factor σ^B, the genetic variation of pressure-resistant subpopulations have helped to elucidate a pressure- protective role of heat shock proteins by the expression of heat inducible genes *hrcA* and *ctsR* named class I and III stress gene repressor in *L. monocytogenes* (Karatzas et al., 2003, 2005; Liu et al., 2012, 2011; Van Boeijen et al., 2010). From proteomic standpoint, pressure-treated *L. monocytogenes* is quite reactive, upregulating 23 proteins, some of them are related to withstand stress conditions and to mechanisms for cell repairing (Jofré et al., 2007). The variable gene expression response at strain level, could be the responsible of the difference between *L. monocytogenes* strains to resist to high hydrostatic pressure.

We found no correlation in pressure resistance and some geno- and phenotypical virulence characteristics of *L.* monocytogenes. Although the relationship between virulence and high hydrostatic pressure has not been previously studied in *L. monocytogenes,* there are several works comparing the pressure-resistance of pathogenic and non-pathogenic *Escherichia coli* strains (Garcia-Hernandez et al., 2015; Liu et al., 2015; Reineke et al., 2014; Sheen et al., 2015). In general, the presence of virulence factors is not related to ability of *E. coli* to withstand high hydrostatic pressure, allowing the selection of non-pathogenic strains as surrogates for validation studies. The origin of the strains was not related to their pressure-tolerance ($p > 0.05$) either. Food-, animal-, and human-isolated strains presented different resistance to high pressure. The origin did not seem to be a good indicator of pressure-resistance. Other authors found no correlation between the origin of the *Listeria monocytogenes* strains and their cold and heat tolerance (Arguedas-Villa et al., 2010; Aryani et al., 2015).

Table 1. Cluster membership of *Listeria monocytogenes* strains according to the pressure-induced inactivation

Strain[a]		Source of isolation[b]	Listeriosis-case history[c]	Lineage[d]	Serogroup[e]	Haemolysis activity[f]	Internalins genes[g]			Inactivation Cluster	Sublethal injury Cluster
Name	***Other denomination***						***inl A***	***inl C***	***inl J***		
ILSI 01	Scott A	Human	Epidemic	I	4	+	+	+	+	**Cluster A**	Cluster X
ILSI 13	FSL M1-004	Human	Sporadic	II	*Listeria* spp	-	+	+	+		
ILSI 17	FSL J1-094	Human	Sporadic	II	2	+	+	+	+		
ITA 360	Noruega 1328	Animal	Sporadic	I*	4	+	+	-	-		
ITA 72	CECT 934	Unknown	Not-related	III*	2	+	+	+	+		
ITA 74	CECT 936	Animal	Sporadic	I*	4	+	+	+	+		
ITA 75	CECT 937	Animal	Sporadic	I*	*Listeria* spp	+	+	-	+		
ITA 76	CECT 938	Food, meat	Not-related	I*	2	-	+	+	+		
ILSI 07	FSL J1-177	Human	Sporadic	I	3	+	+	+	+	**Cluster A**	Cluster Y
ILSI 35	FSL R2-499	Human	Epidemic	II	1	-	+	+	+		
ILSI 09	FSL J1-169	Human	Sporadic	I	3	-	+	+	+	**Cluster A**	Cluster Z
ILSI 18	FSL C1-115	Human	Sporadic	II	1	+	+	+	+		
ILSI 19	FSL J1-031	Human	Sporadic	III	*Listeria* spp	-	+	+	+		
ILSI 31	FSL N3-022	Food, dairy	Epidemic	I	4	-	+	+	+		
ILSI 37	FSL R2-500	Food, dairy	Epidemic	I	4	+	+	+	+		
ILSI 39	FSL R2-502	Food, dairy	Epidemic	I	3	+	-	+	+		
ITA 358	Noruega 144	Human	Sporadic	II*	1	+	+	+	+		
ITA 82	CECT 5725	Food, vegetable	Not-related	III/IV*	4	-	+	+	+		

Table 1. (cont.)

Strain[a]		Source of isolation[b]	Listeriosis-case history[c]	Lineage[d]	Serogroup[e]	Haemolysis activity[f]	Internalins genes[g]			Inactivation Cluster	Sublethal injury Cluster
Name	***Other denomination***						***inl A***	***inl C***	***inl J***		
ILSI 21	FSL W1-111	Unknown	Not-related	IV	*Listeria* spp	+	+	+	-	**Cluster B**	Cluster X
ITA 71	CECT 932	Human	Sporadic	II*	1	-	+	+	+		
ILSI 03	FSL J2-020	Animal	Sporadic	II	1	-	+	+	+	**Cluster B**	Cluster Y
ILSI 04	FSL J1-110	Food, dairy	Epidemic	I	4	+	+	+	+		
ILSI 11	FSL C1-056	Human	Sporadic	II	1	-	+	+	+		
ILSI 27	FSL N3-008	Food, vegetable	Epidemic	I	4	-	+	+	+		
ILSI 29	FSL N3-013	Food, meat	Epidemic	I	4	+	+	+	+		
ILSI 36	FSL N1-227	Food, meat	Epidemic	I	4	-	+	+	+		
ITA 77	CECT 940	Food, vegetable	Not-related	II*	4	-	+	+	+		
ILSI 10	FSL J1-049	Human	Sporadic	I	2	-	+	+	+	**Cluster C**	Cluster Y
ITA 83	UdG 1010	Food, dairy	Not-related	II*	2	+	+	+	+		
L innocua	UBU01	Food, meat	Not-related	N/A	*Listeria* spp	-	-	-	-		
ITA 366	ISS-ROMA CLISS 15	Unknown	Not-related	II	1	+	-	+	+	**Cluster C**	Cluster Z

[a] International Life Science Institute, ILSI; Technical Agriculture Institute of Castilla y León, ITA; University of Burgos, UBU. Spanish Type Culture Collection, CECT.
[b,c] Information provided by the collection owners
[d] Information provided by the collection owners. * Classified according to its serogroup (Nadon et al., 2001)
[e] According to the method by Doumith et al. (2004)
[f] According to the CAMP test
[g] According to the method by Liu et al. (2007b)

Conclusions

The pressure resistance of *L. monocytogenes* in PBS buffer varied between strains depending on the high pressure processing conditions. According to the levels of inactivation and sublethal injury, strains were grouped in clusters, differently affected by pressure and holding time.

Twenty-seven out of thirty strains exhibited a low-intermediate resistance. Three strains were particullary tolerant to high pressure, being candidates to be included in cocktails for use in validation and challenge studies. The strain of *L. innocua* UBU seemed to be a potential candidate as surrogate microorganism in HPP studies. The latter results should be tested and validated.

In the light of this study, HPP processing at 600 MPa for 360 s is able to control the pathogen above 5-log inactivation levels. However, the efficacy should be verified and validated in real food matrices.

The different levels of pressure tolerance of this wide set of strains were not correlated with factors such as lineage, serogroup, virulence factors, pathogenicity or origin. Other genetic and phenotypic characteristics than those covered in this work could be evaluated in further studies for understanding which factors are implicated in the strain variability of pressure-resistance of for designing efficient HPP treatments in order to control successfully the presence of *L. monocytogenes.*

References

Abe, F., 2007. Exploration of the effects of high hydrostatic pressure on microbial growth, physiology and survival: perspectives from piezophysiology. *Biosci. Biotechnol. Biochem*. 71, 2347–2357.

Abu Mraheil, M. 2013. *Listeria*, in: Rosenberg, E., DeLong, E.F., Stackebrandt, E., Thompson, F., Lory, S. (Eds.), the Prokaryotes - Human Microbiology. Springer-Verlag Berlin Heidelberg, pp. 219–235.

Alpas, H., Kalchayanand, N., Bozoglu, F., Ray, B., 2000. Interactions of high hydrostatic pressure, pressurization temperature and pH on death and injury of pressure-resistant and pressure-sensitive strains of foodborne pathogens. *Int. J. Food Microbiol.* 60, 33–42.

Alpas, H., Kalchayanand, N., Bozoglu, F., Sikes, A, Dunne, C.P., Ray, B., 1999. Variation in resistance to hydrostatic pressure among strains of food-borne pathogens. *Appl. Environ. Microbiol*. 65, 4248–4251.

Arguedas-Villa, C., Stephan, R., Tasara, T., 2010. Evaluation of cold growth and related gene transcription responses associated with *Listeria monocytogenes* strains of different origins. *Food Microbiol.* 27, 653–660.

Aryani, D.C., den Besten, H.M.W., Hazeleger, W.C., Zwietering, M.H., 2015. Quantifying variability on thermal resistance of *Listeria monocytogenes. Int. J. Food Microbiol*. 193, 130–138.

Augustin, J.C., Bergis, H., Midelet-Bourdin, G., Cornu, M., Couvert, O., Denis, C., Huchet, V., Lemonnier, S., Pinon, A., Vialette, M., Zuliani, V., Stahl, V., 2011. Design of challenge testing experiments to assess the variability of *Listeria monocytogenes* growth in foods. *Food Microbiol.* 28, 746–754.

Balasubramaniam, V.M., Ting, E.Y., Stewart, C.M., Robbins, J., 2004. Recommended laboratory practices for conducting high-pressure microbial inactivation experiments. *Innov. Food Sci. Emerg. Technol*. 5, 299–306.

Barmpalia-Davis, I.M., Geornaras, I., Kendall, P., Sofos, J.N., 2008. Differences in survival among 13 *Listeria monocytogenes* strains in a dynamic model of the stomach and small intestine. *Appl. Environ. Microbiol*. 74, 5563–5567.

Begot, C., Lebert, I., Lebert, A., 1997. Variability of the response of 66 *Listeria monocytogenes* and *Listeria innocua* strains to different growth conditions. *Food Microbiol*. 14, 403–412.

Benito, A., Ventoura, G., Casadei, M., Robinson, T., Mackey, B., 1999. Variation in resistance of natural isolates of *Escherichia coli* O157 to high hydrostatic pressure, mild heat, and other stresses. *Appl. Environ. Microbiol*. 65, 1564–1569.

Bergholz, T.M., den Bakker, H.C., Fortes, E.D., Boor, K.J., Wiedmann, M., 2010. Salt stress phenotypes in *Listeria monocytogenes* vary by genetic lineage and temperature. *Foodborne Pathog. Dis.* 7, 1537–1549.

Bourdichon, F., 2014. *Listeria monocytogenes*: what have we learned from the last 30 years? New food Mag. Food Saf. Suppl. 17, 3–7.

Bozoglu, F., Alpas, H., Kaletunç, G., 2004. Injury recovery of foodborne pathogens in high hydrostatic pressure treated milk during storage. *FEMS Immunol. Med. Microbiol*. 40, 243–7.

Chaturongakul, S., Raengpradub, S., Wiedmann, M., Boor, K.J., 2008. Modulation of stress and virulence in *Listeria monocytogenes*. *Trends Microbiol*. 16, 388–396.

Chen, H., Neetoo, H., Ye, M., Joerger, R.D., 2009. Differences in pressure tolerance of *Listeria monocytogenes* strains are not correlated with other stress tolerances and are not based on differences in *CtsR*. *Food Microbiol*. 26, 404–408.

Chenal-Francisque, V., Lopez, J., Cantinelli, T., Caro, V., Tran, C., Leclercq, A., Lecuit, M., Brisse, S., 2011. Worldwide distribution of major clones of *Listeria monocytogenes*. *Emerg. Infect. Dis*. 17, 1110–1112.

Crim, S.M., Iwamoto, M., Huang, J.Y., Griffin, P.M., Gilliss, D., Cronquist, A.B., Cartter, M., Tobin-D'Angelo, M., Blythe, D., Smith, K., Lathrop, S., Zansky, S., Cieslak, P.R., Dunn, J., Holt, K.G., Lance, S., Tauxe, R., Henao, O.L., 2014. Incidence and trends of infection with pathogens transmitted commonly through food--Foodborne Diseases Active Surveillance Network, 10 U.S. sites, 2006-2013. *MMWR. Morb. Mortal. Wkly*. Rep. 63, 328–32.

Doumith, M., Buchrieser, C., Glaser, P., Jacquet, C., Martin, P., 2004. Differentiation of the Major *Listeria monocytogenes* Serovars by Multiplex PCR. *J. Clin. Microbiol*. 42, 3819–3822.

Doyle, M.E., Mazzotta, A.S., Wang, T., Wiseman, D.W., Scott, V.N., 2001. Heat Resistance of *Listeria monocytogenes. J. Food Prot*. 64, 410–429.

EFSA, ECDC, 2015. Trends and sources of zoonoses, zoonotic agents and food-borne. *EFSA J.* 13, 1–162.

FDA, 2000. Kinetics of microbial inactivation for alternative food processing technologies: High pressure procesing, Safe Practices for Food Processes.

Francis, G. a., O'Beirne, D., 2005. Variation among strains of *Listeria monocytogenes*: Differences in survival on packaged vegetables and in response to heat and acid conditions. *Food Control* 16, 687–694.

FSAI, 2005. The Control and Management of *Listeria monocytogenes* Contamination of Food. *Food safety authority of Ireland.* Dublin, Ireland.

Gänzle, M., Liu, Y., 2015. Mechanisms of pressure-mediated cell death and injury in *Escherichia coli*: from fundamentals to food applications. *Front. Microbiol*. 6, 1–10.

Garcia-Hernandez, R., Mcmullen, L., Gänzle, M.G., 2015. Development and validation of a surrogate strain cocktail to evaluate bactericidal effects of pressure on verotoxigenic *Escherichia coli. Int. J. Food Microbiol*. 205, 16–22.

Georget, E., Sevenich, R., Reineke, K., Mathys, A, Heinz, V., Callanan, M., Rauh, C., Knorr, D., 2015. Inactivation of microorganisms by high isostatic pressure processing in complex matrices : A review. *Innov. Food Sci. Emerg. Technol*. 27, 1–14.

Health Canada, 2010. Guidance for Industry on Novelty Determination of High Pressure Processing (HPP) -Treated Food Products.

Jasson, V., Uyttendaele, M., Rajkovic, A., Debevere, J., 2007. Establishment of procedures provoking sub-lethal injury of *Listeria monocytogenes*, *Campylobacter jejuni* and *Escherichia coli* O157 to serve method performance testing. *Int. J. Food Microbiol*. 118, 241–249.

Jofré, A., Aymerich, T., Bover-Cid, S., Garriga, M., 2010. Inactivation and recovery of *Listeria monocytogenes*, *Salmonella enterica* and *Staphylococcus aureus* after high hydrostatic pressure treatments up to 900 MPa. *Int. Microbiol*. 13, 105–112.

Jofré, A., Champomier-Vergès, M., Anglade, P., Baraige, F., Martín, B., Garriga, M., Zagorec, M., Aymerich, T., 2007. Protein synthesis in lactic acid and pathogenic bacteria during recovery from a high pressure treatment. *Res. Microbiol*. 158, 512–520.

Kadam, S.R., den Besten, H.M.W., van der Veen, S., Zwietering, M.H., Moezelaar, R., Abee, T., 2013. Diversity assessment of *Listeria monocytogenes* biofilm formation: Impact of growth condition, serotype and strain origin. *Int. J. Food Microbiol.* 165, 259–264.

Karatzas, K., Wouters, J., Gahan, C.G.M., Hill, C., Abee, T., Bennik, M.H.J., 2003. The CtsR regulator of *Listeria monocytogenes* contains a variant glycine repeat region that affects piezotolerance, stress resistance, motility and virulence. *Mol. Microbiol*. 49, 1227–1238.

Karatzas, K., Zervos, A., Tassou, C.C., Mallidis, C.G., Humphrey, T.J., 2007. Piezotolerant small-colony variants with increased thermotolerance, antibiotic susceptibility, and low invasiveness in a clonal *Staphylococcus aureus* population. *Appl. Environ. Microbiol.* 73, 1873–1881.

Karatzas, K.A.G., Valdramidis, V.P., Wells-bennik, M.H.J., 2005. Contingency Locus in *ctsR* of *Listeria monocytogenes* Scott A : a Strategy for Occurrence of Abundant Piezotolerant Isolates within Clonal Populations. *Appl. Environ. Microbiol*. 71, 8390–8396.

Lado, B.H., Yousef, A.E., 2003. Selection and Identification of a *Listeria monocytogenes* Target Strain for Pulsed Electric Field Process Optimization. *Appl. Environ. Microbiol*. 69, 1010-1017.

Lianou, A., Koutsoumanis, K.P., 2013. Strain variability of the behavior of foodborne bacterial pathogens: A review. *Int. J. Food Microbiol.* 167, 310–321.

Liu, D., Lawrence, M.L., Ainsworth, A. J., Austin, F.W., 2007a. Toward an improved laboratory definition of *Listeria monocytogenes* virulence. *Int. J. Food Microbiol*. 118, 101–115.

Liu, D., Lawrence, M.L., Austin, F.W., Ainsworth, A.J., 2007b. A multiplex PCR for species- and virulence-specific determination of Listeria monocytogenes. *J. Microbiol. Methods* 71, 133–40.

Liu, Y., Gill, A., Mcmullen, L., Gänzle, M.G., 2015. Variation in Heat and Pressure resistance of Verotoxigenic and Nontoxigenic *Escherichia coli. J. Food Prot*. 78, 111–120.

Liu, Y., Huang, L., Joerger, R.D., Gunther IV, N., 2012. Genes that are affected in HHP treatments in a *Listeria monocytogenes* Scott A *ctsR* delection mutant. *Microb. Biochem. Technol*. S2:003.

Liu, Y., Ream, A., Joerger, R.D., Liu, J., Wang, Y., 2011. Gene expression profiling of a pressure-tolerant *Listeria monocytogenes* Scott A *ctsR* deletion mutant. *J. Ind. Microbiol. Biotechnol.* 38, 1523–1533.

Luber, P., Crerar, S., Dufour, C., Farber, J., Datta, A., Todd, E.C.D., 2011. Controlling *Listeria monocytogenes* in ready-to-eat foods: Working towards global scientific consensus and harmonization - Recommendations for improved prevention and control. *Food Control* 22, 1535–1549.

Mohamed, H.M.H., Diono, B.H.S., Yousef, A.E., 2012. Structural changes in *Listeria monocytogenes* treated with gamma radiation, pulsed electric field and ultra-high pressure. J. Food Saf. 32, 66–73

NACMCF, 2010. Parameters for determining inoculated pack/challenge study protocols. *J. Food Prot.* 73, 140–202.

NACMCF, 2005. Considerations for establishing safety-based consume-by date labels for refrigerated ready-to-eat foods. *J. Food Prot*. 68, 1761–1775.

Nadon, C., Woodward, D.L., Young, C., Rodgers, F.G., Wiedmann, M., 2001. Correlations between molecular subtyping and serotyping of *Listeria monocytogenes. J. Clin. Microbiol.* 39, 2704–2707.

Orsi, R.H., Bakker, H.C.D., Wiedmann, M., 2011. *Listeria monocytogenes* lineages: Genomics, evolution, ecology, and phenotypic characteristics. *Int. J. Med. Microbiol*. 301, 79–96.

Rasch, M., Knøchel, S., 1998. Variations in tolerance of *Listeria monocytogenes* to nisin, pediocin PA-1 and bavaricin A. *Lett. Appl. Microbiol*. 27, 275–278.

Reineke, K., Sevenich, R., Hertwig, C., Janßen, T., Fröhling, A., Knorr, D., Wieler, L.H., Schlüter, O., 2014. Comparative study on the high pressure inactivation behavior of the Shiga toxin-producing *E. coli* O104:H4 and O157:H7 outbreak strains and a non-pathogenic surrogate. *Food Microbiol*. 46, 184–194.

Ritz, M., Tholozan, J. L., Federighi, M., Pilet, M. F., 2002. Physiological damages of *Listeria monocytogenes* treated by high hydrostatic pressure. *Int. J. Food Microbiol*. 79, 47–53.

Ritz, M., Tholozan, J.L., Federighi, M., Pilet, M.F., 2001. Morphological and Physiological Characterization of *Listeria monocytogenes* Subjected to High Hydrostatic Pressure. *Appl. Environ. Microbiol.* 67, 2240–2247.

Santillana-Farakos, S.M., Zwietering, M.H., 2011. Data analysis of the inactivation of foodborne microorganisms under high hydrostatic pressure to establish global kinetic parameters and influencing factors. *J. Food Prot.* 74, 2097–2106.

Sheen, S., Cassidy, J., Scullen, B., Sommers, C., 2015. Inactivation of a diverse set of Shiga toxin-producing *Escherichia coli* in ground beef by high pressure processing. *Food Microbiol*. 52, 84–87.

Shimasaki, N., Kiyohara, T., Totsuka, A, Nojima, K., Okada, Y., Yamaguchi, K., Kajioka, J., Wakita, T., Yoneyama, T., 2009. Inactivation of hepatitis A virus by heat and high hydrostatic pressure : variation among laboratory strains. *Vox Sanguinis* 96 (1) 14–19.

Simpson, R., Gilmour, A., 1997a. The effect of high hydrostatic pressure on *Listeria monocytogenes* in phosphate-buffered saline and model food systems. *J. Appl. Microbiol.* 83, 181–188.

Simpson, R., Gilmour, A., 1997b. The resistance of *Listeria monocytogenes* to high hydrostatic pressure in foods. *Food Microbiol.* 14, 567–573.

Tay, A., Shellhammer, T.H., Yousef, A.E., Chism, G.W., 2003. Pressure death and tailing behavior of *Listeria monocytogenes* strains having different barotolerances. *J. Food Prot.* 66, 2057–2061.

Tholozan, J.L., Ritz, M., Jugiau, F., Federighi, M., Tissier, J.P., 2000. Physiological effects of high hydrostatic pressure treatments on *Listeria monocytogenes* and *Salmonella typhimurium. J. Appl. Microbiol.* 88, 202–212.

USDA-FSIS, 2014. FSIS Compliance Guideline: Controlling *Listeria monocytogenes* in Post-lethality Exposed Ready-to-Eat Meat and Poultry Products January 2014, 143.

Van Boeijen, I.K.H., Chavaroche, A., Valderrama, W.B., Moezelaar, R., Zwietering, M.H., Abee, T., 2010. Population diversity of *Listeria monocytogenes* LO28: Phenotypic and genotypic characterization of variants resistant to high hydrostatic pressure. *Appl. Environ. Microbiol*. 76, 2225–2233.

Van Boeijen, I.K.H., Moezelaar, R., Abee, T., Zwietering, M.H., 2008. Inactivation kinetics of three *Listeria monocytogenes* strains under high hydrostatic pressure. *J. Food Prot*. 71, 2007–13.

Van Der Veen, S., Moezelaar, R., Abee, T., Wells-Bennik, M.H.J., 2008. The growth limits of a large number of *Listeria monocytogenes* strains at combinations of stresses show serotype- and niche-specific traits*. J. Appl. Microbiol*. 105, 1246–1258.

Waite-Cusic, J.G., Diono, B.H.S., Yousef, A.E., 2011. Screening for *Listeria monocytogenes* surrogate strains applicable to food processing by ultrahigh pressure and pulsed electric field. *J. Food Prot.* 74, 1655–61.

Wells-Bennik, M.H.J., Karatzas, K.A.G., Moezelaar, R., Abee, T., 2008. *Listeria monocytogenes*: High hydrostatic pressure resistance and survival strategies, in:

Michiels, C., Bartlett, D., Aersten, A. (Eds.), High-Pressure Microbiology. ASM Press, Washington D. C., pp. 101–116.

Wemekamp-Kamphuis, H.H., Wouters, J., Leeuw, P., De Hain, T., Chakraborty, T., Abee, T., 2004. Identification of Sigma Factor B -Controlled Genes and Their Impact on Acid Stress, High Hydrostatic Pressure, and Freeze Survival in *Listeria monocytogenes* EGD-e. *Appl. Environ. Microbiol.* 70, 3457–3466.

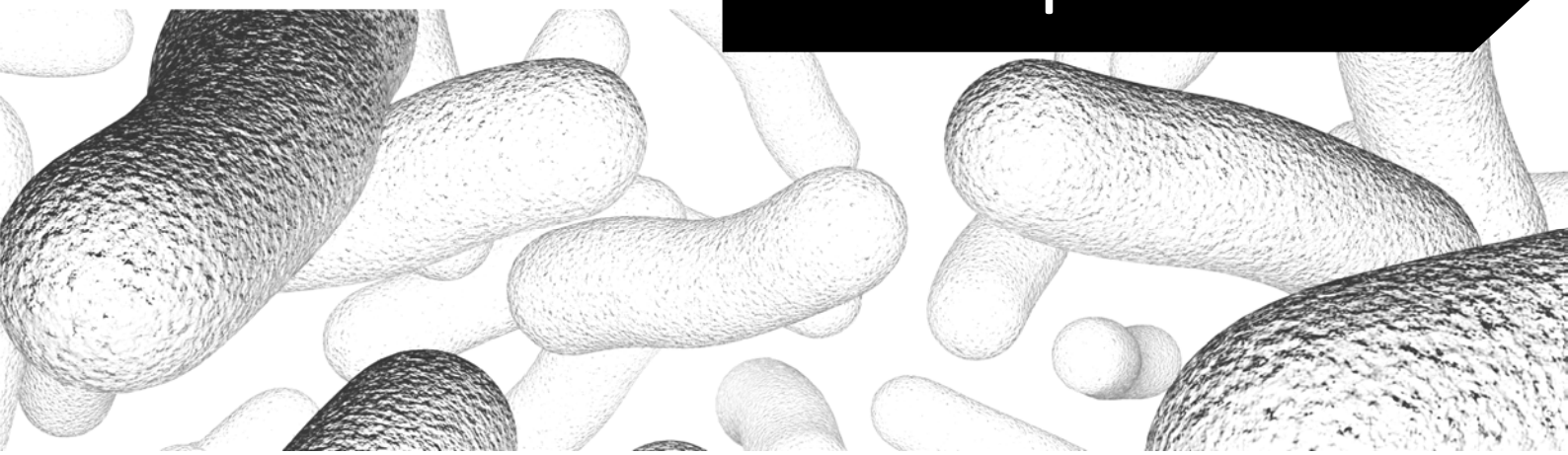
Youart, A.M., Huang, Y., Stewart, C.M., Kalinowski, R.M., Legan, J.D., 2010. Modeling time to inactivation of *Listeria monocytogenes* in response to high pressure, sodium chloride, and sodium lactate. *J. Food Prot*. 73, 1793–1802.

Zhu, M., Du, M., Cordray, J., Ahn, D.U., 2005. Control of *Listeria monocytogenes* contamination in ready-to-eat meat products. *Compr. Rev. Food Sci. Food Saf*. 4, 34–42.

Diversity of *Listeria monocytogenes* strains response to high hydrostatic pressure.

A case study in RTE meat products

Chapter 2



Chapter 2

Diversity of *Listeria monocytogenes* strains response to high hydrostatic pressure. A case study in RTE meat products

Introduction

Prior to the 1980s, listeriosis was a rarity, only associated to an animal disease. Since then, the number of cases on the association of *Listeria monocytogenes* isolates with human disease increased, and the microorganism started to be considered one of the major foodborne pathogens because its high morbidity and mortality regardless its moderate prevalence in foods (Bourdichon, 2014). The annual estimation of illnesses worldwide caused by *L. monocytogenes* is 23,150 cases, which led to 5463 deaths globally (de Noordhout et al., 2014). In Europe during 2013, it was reported 1,763 confirmed cases of listeriosis in people across the continent, indicating 0.44 cases per 100,000 population. A total of 191 deaths were reported during the same period, reaching a case-fatality rate of 15.6%, being the highest rate among all zoonosis surveyed in Europe (EFSA and ECDC, 2015).

After major outbreaks in North America and Europe during the 1980s, the significance of foods as the primary route of transmission of the bacteria was recognized. The vast majority of cases of human listeriosis has been associated to the consumption of foods that do not meet the microbiological criteria for *L. monocytogenes* in foods, whether that standard is zero-tolerance or 100 CFU/g (FAO, 2004). According to the International Life Science Institute Risk Science Institute Expert Panel, the high-risk foods for carrying the pathogen: support its growth to high numbers, are ready-to-eat (RTE) foods, require refrigeration, and are stored for an extended period of time (ILSI, 2005). Among meat

products, RTE deli meats and non-reheated frankfurters are considered high risk listeriosis-associated foods, while fermented sausages and meat products which required a post-heating procedure were classified as moderate risk products. Dry meat products such as salami and dry-cured ham were not considered the greatest risk because their composition does not support the growth of the pathogen, however, they can harbor the bacteria (Baer et al., 2013).

During 1992-2012 period, RTE sliced cooked meat products have been associated to 11 listeriosis outbreaks in Europe, North America and Oceania (NSW food authority, 2013). In The Netherlands during 2009, the cost-of-illness in meat-borne listeriosis was estimated in € 0.5 million, a 25 % of the total listeriosis-associated cost (Haagsma et al., 2009). The presence of *L. monocytogenes* in cooked meat products is most likely to be the result of recontamination following processing, which may occur during additional handling such as peeling, slicing, and repackaging (Lianou and Sofos, 2007). Dry-cured meat products shows a low water activity (a_w) usually lower than 0.92 and high salt concentration (higher than 4 %), which hardly support the growth of *L. monocytogenes*. In fact, dry-cured or dry-fermented meat products have never been involved in listeriosis outbreak. However, the presence of the pathogen constitutes an impediment of export to those countries with a zero-tolerance policy such as USA, Japan and Australia (Bover-Cid et al., 2011). In Australia, dry-cured ham from Spain and Italy failed eight times during 2010 – 2012 at the border due to the presence of the microorganism (NSW food authority, 2013). Therefore, the presence of *L. monocytogenes* must be controlled using a postlethality treatment to avoid human disease in RTE cooked meat products and food recalls in case of cured meats.

Technologies such as surface pasteurization, E-beam radiation, UV-light, addition of chemical preservatives, and biopreservation by using protective cultures and bacteriocins have been demonstrated a good performance to control de pathogen in RTE meat products (Baer et al., 2013; Jiang and Xiong, 2014; Zhu et al., 2005). High pressure processing (HPP) stands out among other preservation technologies because it has the potential to inactivate pathogenic and spoilage microorganisms, and to meet the consumer demands in relation to high quality foods (Bover-Cid et al., 2011; Campus,

2010; Garriga et al., 2004). HPP technology is efficient to control *L. monocytogenes* in RTE meat products such as cooked ham (Hereu et al., 2012; Jofré et al., 2009; Koseki et al., 2007) and dry-cured meat meat (Bover-Cid et al., 2015; Hereu et al., 2012; Stollewerk et al., 2012).

Any post-lethality technology must be a part of the establishments' HACCP plan and its effectiveness incorporated into their HACCP plan must be technically-validated and supported by science-based approach. Challenge tests are one of the major types of scientific and technical support documents used to satisfy the design element of HACCP System validation (USDA-FSIS, 2015). These tests involve the inoculation of a product with relevant microorganism(s) and storing under a range of controlled environmental conditions in order to assess the risk of food poisoning or to establish product stability (Betts, 2010). Besides the considerations about processing (e.g. pressure, holding time, come-up time, depressurization time, temperature), the characteristics of the strains used are relevant to obtain reliable information from challenge tests about high pressure technology (Balasubramaniam et al., 2004; NACMCF, 2010).

An assumption frequently made by food microbiologists is that strain-to-strain variation of microbial behavior is equal to or smaller than the experimental variation, and, it is not necessary to be determined and characterized (Whiting and Golden, 2002). However, some authors demonstrated large strain-to-strain variations making the selection of the strains critical in challenge testing (Lianou and Koutsoumanis, 2013). In this case, strains with above average (but no unrealistic) resistance to lethal treatments or those associated with outbreaks are preferred to be included in challenge testing and validation studies (Balasubramaniam et al., 2004).

Aryani et al. (2015) demonstrated that the strain variability in the thermal resistance of *L. monocytogenes* was ten times higher than the experimental variability (the variability between parallel experimental replicates) and four times higher than the biological variability (variability between biologically independent reproductions). Although strain variability in tolerance to high hydrostatic pressure has not been quantified, some studies have reported wide pressure resistance among strains in a same species in lab media (Alpas et al., 1999; Benito et al., 1999; Garcia-Hernandez et al., 2015; Liu et al.,

2015). Regarding *L. monocytogenes*, some strains have shown a higher tolerance than others in buffer and food models (Alpas et al., 1999; Chen et al., 2009; Simpson and Gilmour, 1997a; Tay et al., 2003).

In the previous chapter, we found that the strains of the pathogen exhibited a varied resistance to high hydrostatic pressure in PBS buffer, identifying some pressure-resistant strains. However, the efficacy of the HPP technology concerning microbial inactivation is also dependent on the food matrix, which can exert a synergetic or protective effect with high pressure (Georget et al., 2015; Rendueles et al., 2011). Therefore, in this study, we evaluated individually, the variability in pressure-resistance of fifteen *L. monocytogenes* strains, using cooked and cured ham as model meat matrices. The meat matrices were chosen based on their association to *Listeria*-positive cases according to RASFF (2015) or their economic impact in Spanish meat industry (Cruz, 2013).

Materials and methods

Strains included in this study

Fifteen *L. monocytogenes* strains from three collections, International Life Science Institute – North America (ILSI, USA), Technical Agriculture Institute of Castilla y León (ITACyL, Spain) and University of Burgos (UBU, Spain) were evaluated. These strains were selected according to the pressure resistance exhibited in the previous study and/or their link with meat products associated to human listeriosis. A *L. innocua* strain from UBU collection was included in this study, due to high pressure tolerance exhibited in PBS buffer. As a preliminary step, PCR serogrouping, internalins presence and a biochemical characterization were done according to the methodology described below.

Culture preparation

The stock cultures were kept frozen at −80 °C in 70 % brain heart infusion (BHI) broth (Oxoid, UK) and 30 % glycerol (v/v) (Sigma Aldrich). From the stock culture, a streak of each strain was made onto a tryptone soy agar and 0.6 % yeast extract (TSAYE, Oxoid, UK) plate and incubated for 24 h at 37 °C. A single colony was inoculated in a test tube

pre-filled with 5 mL of BHI broth and incubated until the stationary phase at 37 °C during 18 h. This procedure was done for obtaining cells for molecular biology methodologies as well as for meat product inoculation.

DNA extraction

The DNA used for PCR assays was extracted from 2 mL of cell culture using the GenElute^{TM} Kit (Bacterial Genomic DNA Kit, Sigma, USA) according to the manufacturer's recommendations for Gram-positive bacteria. The DNA obtained was resuspended in 100 µL of the recommended buffer. The extracted DNA was quantified using an Epoch Bioteck spectrophotometer and the GEN 5 v2.00 software (Biotek, Vermont, USA).

PCR serogrouping and presence of internalins genes

Serogrouping of the *L. monocytogenes* strains was done following the protocol by Doumith et al. (2004) with some variations. Multiplex PCR amplifications were performed in a final sample volume of 25 µL containing 1× PCR reaction buffer without $MgCl_2$ (Sigma), 200 µM of each dNTP (dATP, dCTP, dGTP, dUTP) (Promega, Madison, WI), 1 µM of *lmo0737*, ORF2110 and ORF2819; 1.5 µM of *lmo1118* and 0.2 µM of *prs* primer, 2 mM of $MgCl_2$, 0.5 U of *Taq* DNA Polymerase and 100 ng of template DNA. All material was provided by Sigma (Spain).

Internalins genes, *inlA, inlC* and *inlJ*, were detected in each strain according to the method designed by Liu et al. (2007). PCR was conducted in a volume of 25 µL containing 0.8 U *Taq* DNA polymerase 1× PCR buffer, 200 µM dNTPs and 10 ng each *Listeria monocytogenes* DNA, together with 40 pmol each *inlA*, 30 pmol each *inlC* and 20 pmol each *inlJ* primers. All material was provided by Sigma (Spain).

All conventional PCR reactions were performed using a Mastercycler gradient thermal cycler (Eppendorf, Spain). The PCR products were separated by electrophoresis on a 2.0 % Electran agarose gel (VWR, UK) and stained with ethidium bromide (Amresco, USA).

Biochemical characterization

All strains were further subjected to haemolysis on Christie, Atkins, Munch Petersen (CAMP) test on Columbia blood agar (Oxoid, UK) and detection of phosphatidylinositol-

specific phospholipase C (PI-PLC) activity on agar *Listeria* according to Ottaviani and Agosti (ALOA) medium (Oxoid, UK).

Meat products

Each product, cooked and dry-cured ham, was purchased in a local supermarket, 24 h before the inoculation step and stored at 4 ^{o}C until inoculation. Physicochemical parameters, pH and water activity, were analysed using a puncture pH-meter (CRISON, Spain) and Aqua-Lab CX2 equipment (Decagon, US), respectively. The characteristics of sliced cooked ham were a_w = 0.969 ± 0.001 and pH = 5.97 ± 0.03. For dry-cured ham were a_w = 0.908 ± 0.013 and pH = 5.39 ± 0.03.

Inoculation

Two-hundred and fifty microliters of each *L. monocytogenes* strain inoculum were added and streaked on the surface of 25 g ± 0.5 g of each product, obtaining an initial population of approximately 10^7 CFU/g. The sample was packaged in Stomacher bags (Interscience, France). Stomacher bags were put into another plastic bag and were vacuum packaged and kept under refrigeration overnight at 4 ^{o}C until high pressure processing.

High pressure processing conditions

For each meat product, different holding times were tested. For cooked ham, treatments at 600 MPa during 3, 180 and 240 s were done. Dry-cured ham was processed at 600 MPa at 180, 240 and 540 s. All trials were done at room temperature (20 ^{o}C) in a 135-L high pressure unit (Hiperbaric 135 from Hiperbaric S. A., Burgos, Spain), using water as pressurization liquid. The pressure build-up time was 170 s and depressurization time was less than 3 s. The pressure and the range of holding time was selected to cover usual industrial conditions for this type of product. After high pressure processing, the samples were stored at 4 ^{o}C (less than 2 h) until microbiological analysis.

Microbiological analysis

Ten-fold serial dilutions were made in Ringer's solution (Oxoid, UK) and depending on the expected counts the adequate decimal dilution was spread manually on

Chromogenic Listeria Agar (OCLA; Oxoid, UK) and incubated at 37 °C for 48 h. The results were expressed in $\log_{10}$ colony-forming units (CFU)/g.

Data analysis

All strains were analyzed in triplicate for each HPP treatment. Cluster analysis on the complete dataset was performed by an euclidic distance analysis using the Ward method on the non-scaled data. Principal component analysis (PCA) was done on the covariance matrix and two components were extracted. Cluster and PCA analyses were done by using OriginPro 9.0 (Originlab, USA).

Statistically significant differences between clusters according to the characteristics of the strains (lineage, serogroup, virulence, pathogenicity or the source of isolation) were identified by analysis of variance (Statgraphics Centurion XVI, StatPoint Technologies, Inc., USA). All statistical procedures were done on inactivation expressed in $\log_{10}$ colony-forming units (CFU)/mL.

Results and Discussion

Intra-species differences concerning inactivation by HPP in cooked ham and dry-cured ham

The distributions of the inactivation values of pressure-treated *Listeria monocytogenes* in cooked and dry-cured ham are shown in **Figure 1** and **Figure 2**, respectively. In both meat matrices, the range of inactivation depended on the processing conditions applied. In cooked ham, the strains of the pathogen were inactivated 1.8 – 3.9 log CFU/g, 3.6 – 4.9 log CFU/g and 3.7 – 5.3 log CFU/g at 600 MPa during 3, 180 and 360 s, respectively. The range of inactivation levels agreed with those reported by Koseki et al. (2007) for the cooked ham. The inactivation ranges for dry-cured ham were 1.7 – 3.4 log CFU/g, 3.2 – 4.6 log CFU/g and 3.2 – 5.8 log CFU/g when it was processed at 600 MPa for 180, 360 and 540 s, respectively. Our results at 600 MPa, 360 s are in agreement with the findings of Bover-Cid et al. (2015) and Hereu et al. (2012), however, at the longest holding time (540 s), the *L. monocytogenes* inactivation levels reached were higher (around 3-log inactivation) than those reported by Bover-cid et al. (2011) at the same

HPP conditions. The differences between our results and the available previous studies seemed to be due to the different physicochemical characteristics of the dry-cured ham (Dry-cured ham used in this study a_w = 0.908, while Bover-Cid et al. (2011) a_w = 0.88). This lower inactivation values are related to the baroprotective effect exerts by lower values of water activity on the resistance of *L. monocytogenes* (Koseki and Yamamoto, 2007).

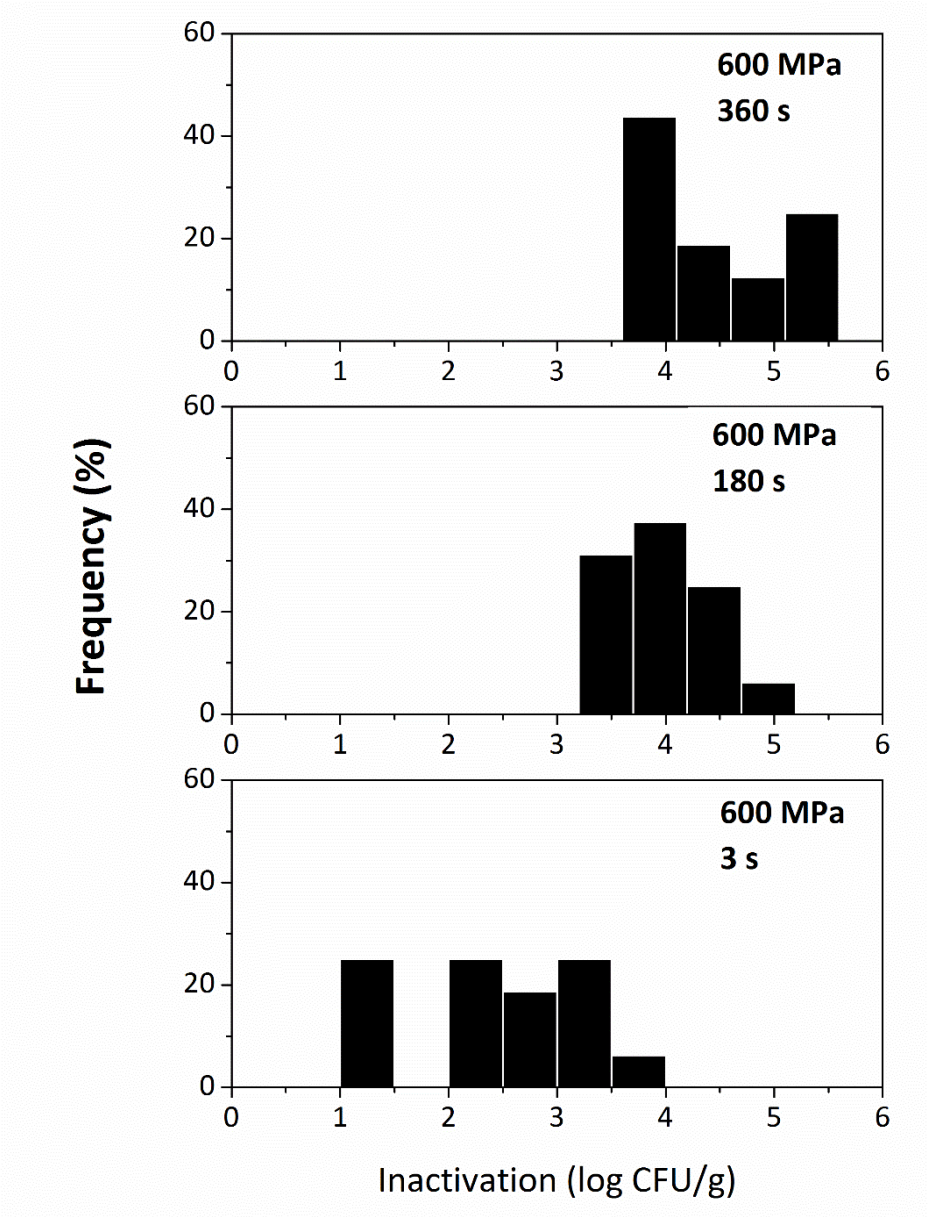


Figure 1. Distribution of inactivation (log CFU/g) of the 15 *Listeria monocytogenes* strains in cooked ham (a_w = 0.969 ± 0.001, pH = 5.97 ± 0.03) by high hydrostatic pressure at 600 MPa, 3 s, 180 s and 360 s.

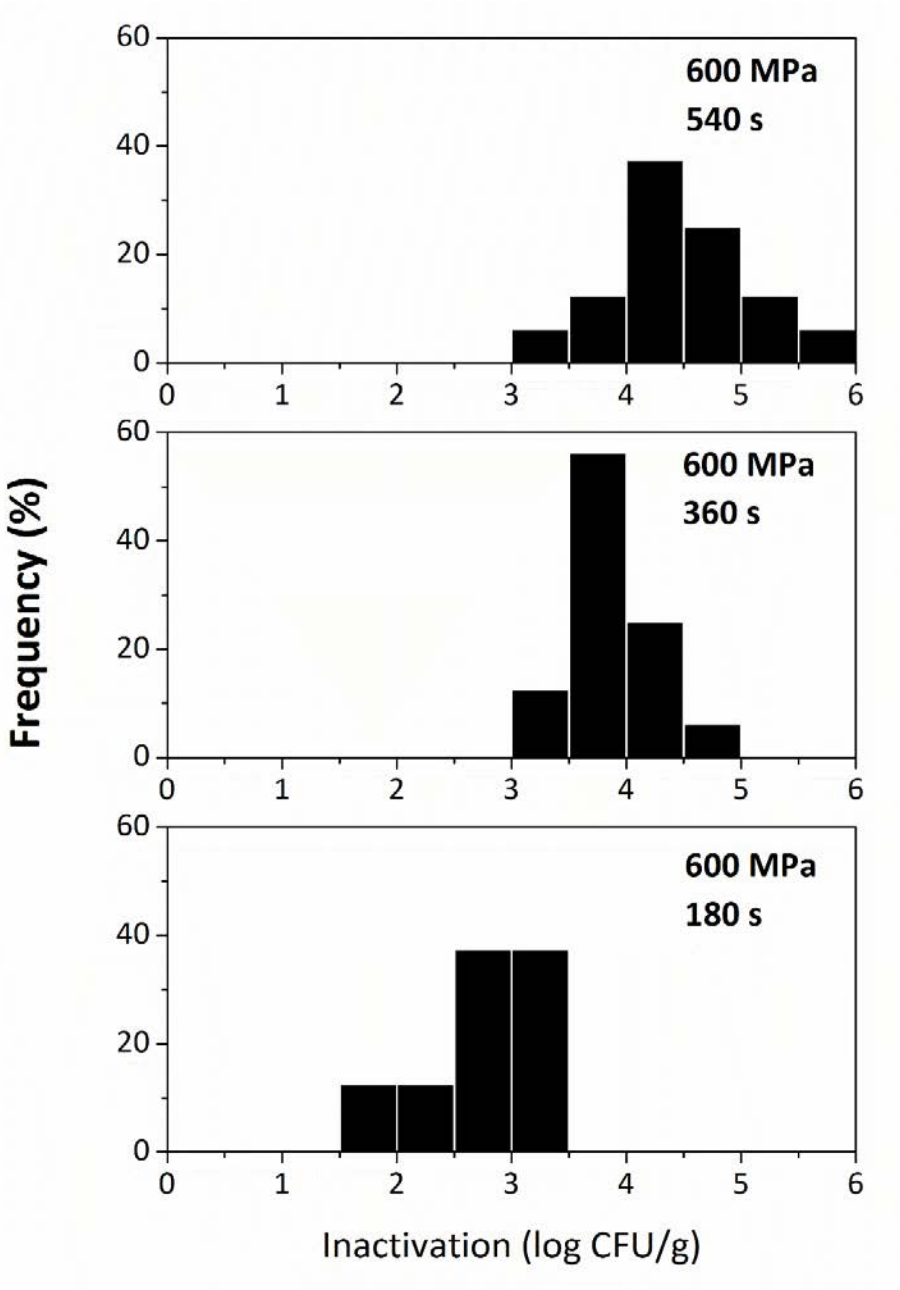


Figure 2. Distribution of inactivation (log CFU/g) of the 15 *Listeria monocytogenes* strains in dry cured ham (a_w = 0.908 ± 0.013, pH = 5.39 ± 0.03) by high hydrostatic pressure at 600 MPa, 180 s, 360 s and 540 s.

Although the impact of food matrix on pressure-resistance of microorganisms has been extensively documented (Georget et al., 2015), the studies about how the meat matrix affects the pressure-tolerance of *L. monocytogenes* at strain level are scarce. Simpson and Gilmour (1997b) found that the inactivation of the pathogen by high hydrostatic pressure was different between the three strains tested, but also the strain-related

variation depended on the concentrations of bovine serum albumin, glucose or lipids in the model food system. In raw and cooked chicken mince, the same authors found that two strains presented around 2 log CFU/g of difference in the tolerance to high pressure (345 MPa), whereas their behavior was similar when the tests were done in cooked beef. Other microorganisms such as *Escherichia coli* and the species of *Campylobacter* genus showed different strain-dependent tolerance to high hydrostatic pressure in meat matrices (Garcia-Hernandez et al., 2015; Liu et al., 2015; Martínez-Rodriguez and Mackey, 2005; Sheen et al., 2015). The differences between pressure-resistant and sensitive ranged between 2 and 3 log cycles for the evaluated microorganisms. The pressure-resistance variability of *L. monocytogenes* has been observed in other food matrices such as HPP-treated whey (Waite-Cusic et al., 2011). Processing at 500 MPa for 60 s achieved 3 log inactivation in the most resistant strains, while the most sensitive were inactivated 5 log CFU/mL.

Besides the ability to withstand high hydrostatic pressure, the microorganism exhibits strain-to-strain differences in growth rate in meat and turkey slurry (Pal et al., 2008) and cooked ham model (Begot et al., 1997). Meat isolated strains have exhibited variable growth at cold temperatures (Arguedas-Villa et al., 2010) and in high osmotic conditions (Bergholz et al., 2010). The latter could explain the strain diversity of the bacteria found in meat products and in processing plants (Martín et al., 2014). The thermal resistance of *L. monocytogenes* showed to be strain-dependent (Aryani et al., 2015) and this variability increased with the decrease in temperature. Our results suggested that pressure-resistance variability depended on the holding time at 600 MPa as well as on the meat matrix. Variance values of inactivation in cooked ham were 0.69, 0.17 and 0.34 at 600 MPa for 3, 180 and 540 s, respectively, indicating a higher variability at the least intense conditions. On the contrary, the variance of inactivation in dry-cured ham was slightly higher at the most intense conditions (0.30, 0.18 and 0.39 at 600 MPa during 180, 360 and 540 s, respectively).

Grouping of *L. monocytogenes* strains according to their different pressure-resistance in cooked ham and in cured ham

Since the strains of the bacterium presented a diverse resistance at the tested conditions, we carried out a clustering analysis for grouping those strains according to their pressure resistance in both meat matrices, analyzed separately. The fifteen strains of *L. monocytogenes* and the strain of *L. innocua* were grouped in three groups Cluster Ch-A (n = 6), Cluster Ch-B (n = 7) and Cluster Ch-C (n = 3), according to their inactivation data in cooked ham (**Figure 3**).

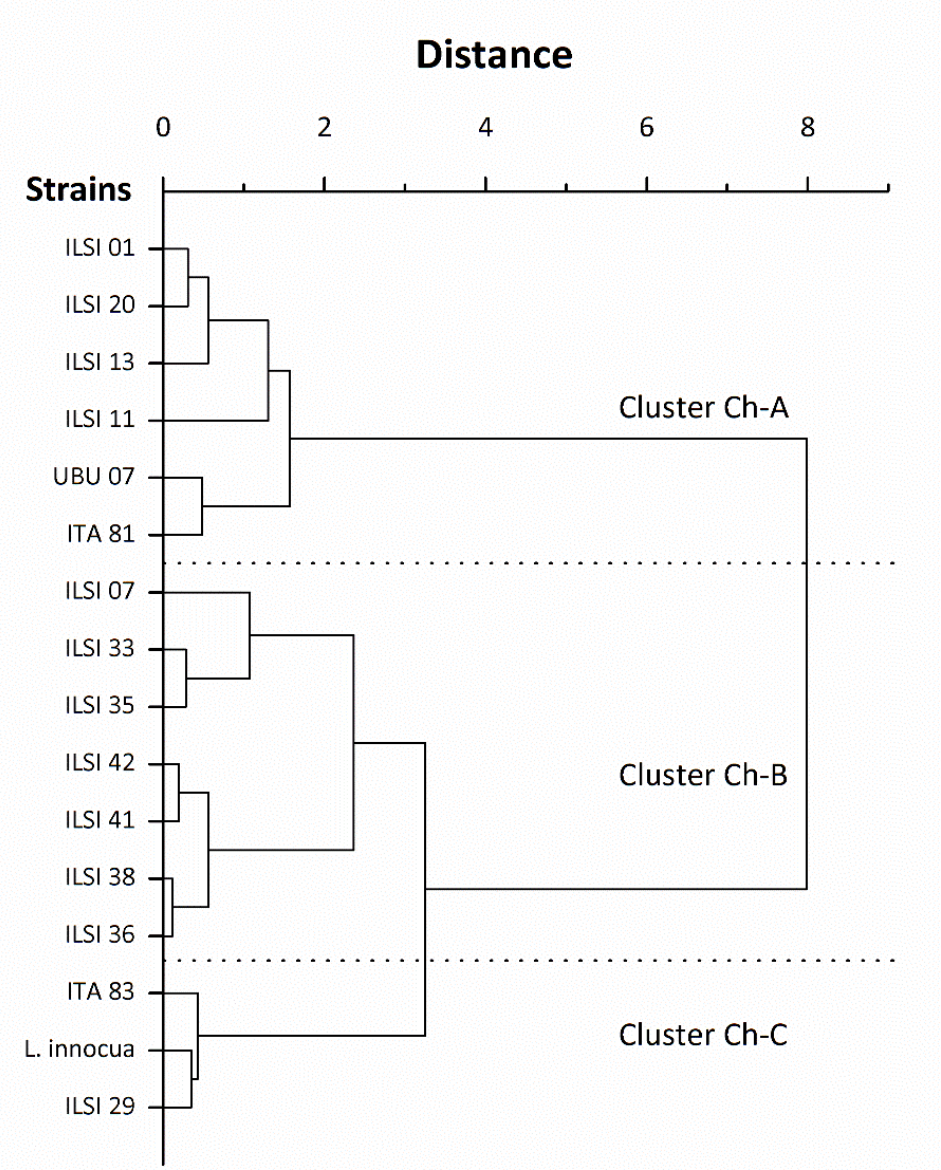


Figure 3. Cluster dendrogram of the inactivation of 15 *Listeria monocytogenes* strains and one *L. innocua* by high hydrostatic pressure in cooked ham (a_w = 0.969 ± 0.001, pH = 5.97 ± 0.03). The strains were clustered by using euclidic distance and Ward grouping method.

Cluster Ch-A was the most pressure-sensitive group, reaching the highest inactivation levels at all conditions ($p < 0.01$) and Cluster Ch-B and Ch-C presented similar mean values ($p > 0.05$) of inactivation at 180 s and 360 s (**Figure 4**). However, cluster Ch-C was significantly ($p < 0.01$) more pressure-resistant (1.3 ± 0.2 log CFU/g) than cluster Ch-B (2.3 ± 0.4 log CFU/g) at 600 MPa during 3 s. According to our results, the pressure-resistance was Ch-C > Ch-B > Ch-A.

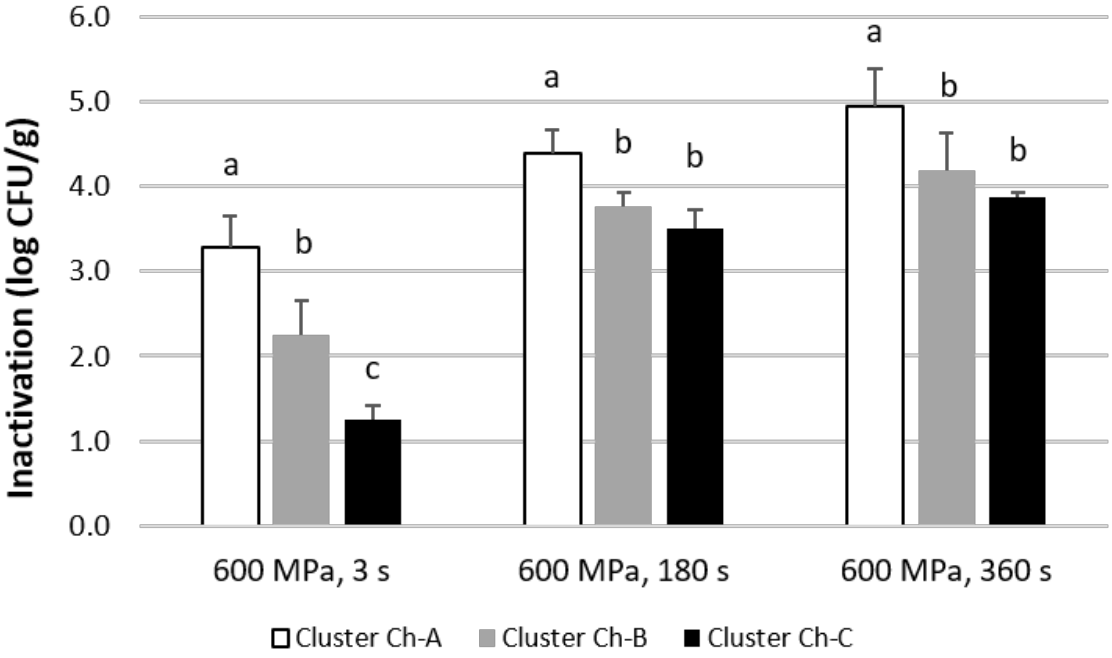


Figure 4. Effect of high pressure processing on inactivation of the three *L. monocytogenes* clusters in cooked ham (Ch; a_w = 0.969 ± 0.001, pH = 5.97 ± 0.03) after pressurization at 600 MPa. Data are represented as mean ± SD. Different letters (a, b , c) at the same pressure and holding time indicate significant differences ($p < 0.05$) based on LSD test of significance.

In dry-cured ham, the multivariate analysis classified the strains in three clusters, as well (**Figure 5**). Cluster Dh-A (n = 6) was more pressure-resistant at the three tested holding times ($p < 0.01$) than cluster Dh-B (n = 7) and Dh-C (n = 3). At 600 MPa for 180 s, there was no statistical differences between the inactivation levels of clusters Dh-B and Dh-C ($p > 0.05$), but when holding time increased, cluster Dh-B exhibited a slight (less than 1 log cycle) but significant ($p < 0.01$) higher tolerance that Dh-C (**Figure 6**). The tolerance to high hydrostatic pressure was Dh-A > Dh-B > Dh-C.

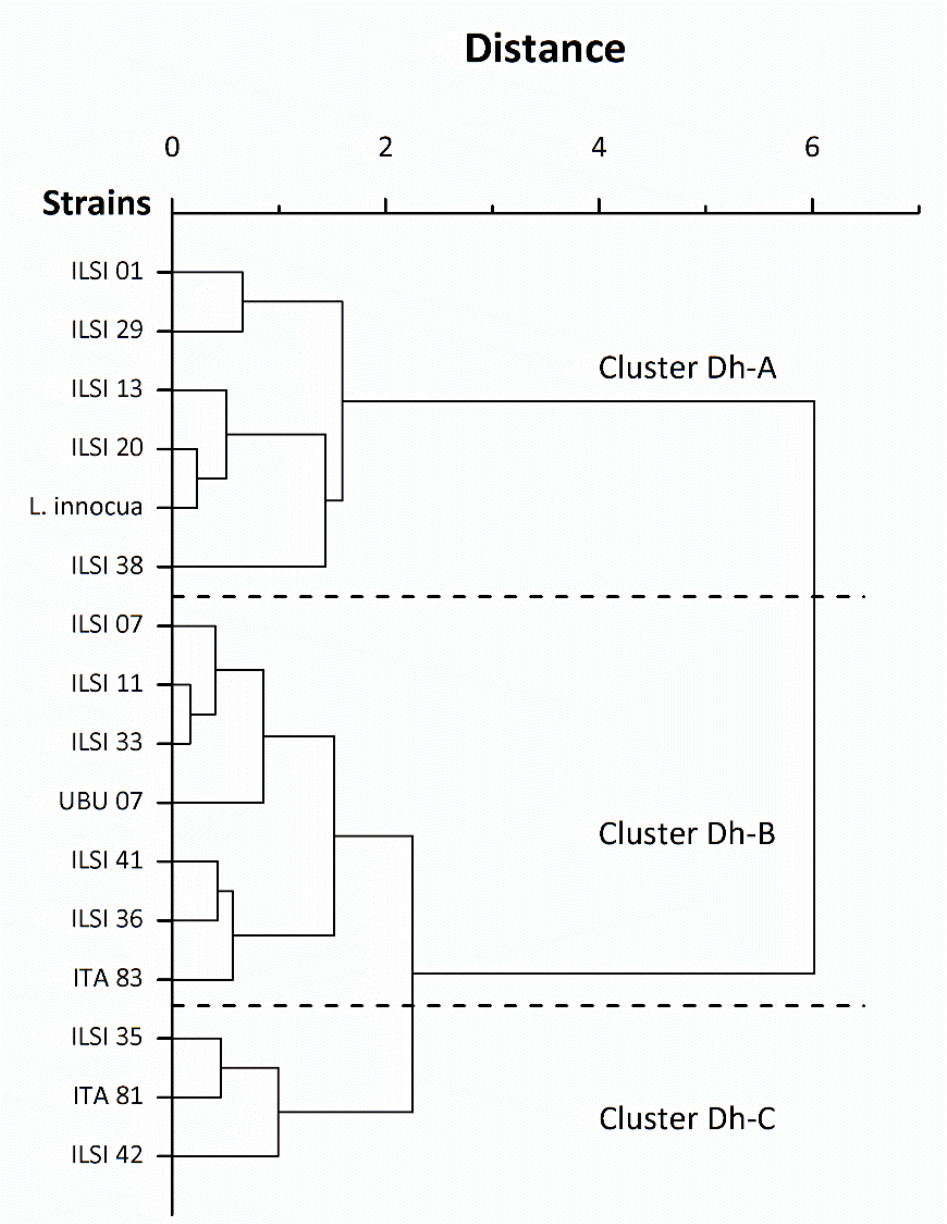


Figure 5. Cluster dendrogram of the inactivation of 15 *Listeria monocytogenes* strains and one *L. innocua* by high hydrostatic pressure in dry-cured ham (a_w = 0.908 ± 0.013, pH = 5.39 ± 0.03). The strains were clustered by using euclidic distance and Ward grouping method.

The increase of holding time led to an increment in the lethal effect of high hydrostatic pressure in all clusters and both meat products (**Figure 4** and **Figure 6**). Interestingly, the more pressure resistant the cluster was, the higher inactivation induced an extension of holding time. In cooked ham, 2.3, 1.5 and 1.1 log-inactivation of difference in cluster Ch-C, Ch-B and Ch-A was achieved, respectively, between 3 and 180 s. A similar result was obtained in dry-cured ham, where a longer holding time, from 180 to 360 s, led to an

increase of 1.3, 0.9 and 1.1 log-inactivation for cluster Dh-A, Dh-B and Dh-C, respectively. However, the increment of inactivation levels from 180 to 360 s in cooked ham, and from 360 to 540 s in dry-cured ham was lower (less than 0.7 log CFU/g) for all clusters. This phenomenon has already been described and is known as tailing, where exposure during longer periods at high pressure does not result in a proportional inactivation (Tay et al., 2003). The presence of pressure-resistant subpopulation of cells explains this effect seen in pure cultures of *L. monocytogenes* subjected to high pressure, where the survivors have exhibited higher tolerance than the wild-type strain (Karatzas et al., 2007; Van Boeijen et al., 2010).

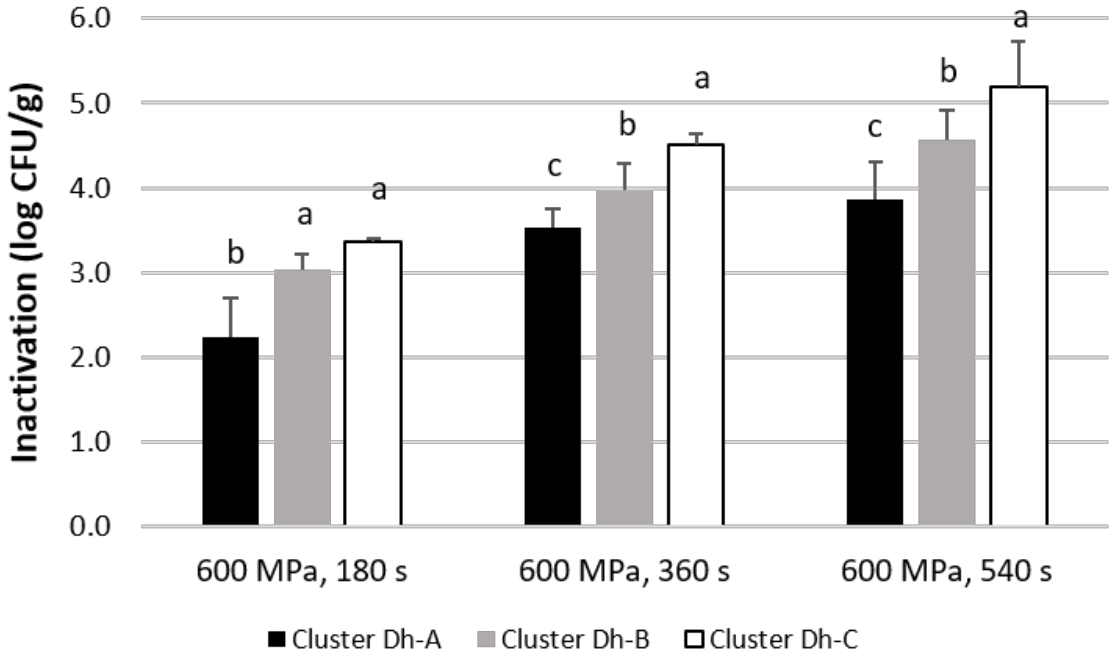


Figure 6. Effect of high pressure processing on inactivation of the three *L. monocytogenes* clusters in dry-cured ham (Dh; a_w = 0.908 ± 0.013, pH = 5.39 ± 0.03) after pressurization at 600 MPa. Data are represented as mean ± SD. Different letters (a, b, c) at the same pressure and holding time indicate significant differences ($p < 0.05$) based on LSD test of significance.

Several food safety authorities have recognized the use of high hydrostatic pressure technology for controlling the presence of *L. monocytogenes* in RTE meat products (FSAI, 2005; FSIS, 2014; Health Canada, 2006). However, those institutions demand validation studies for demonstrating the suitability of the technology in any particular RTE meat product before being commercialized. In USA, any post-lethality treatment expects to

achieve 2-log-inactivation or greater, whereas in Canada, the goal is 3 log-inactivation (FSIS, 2014; Health Canada, 2010). Based on the results of inactivation of the most resistant cluster, we determined that 600 MPa for 180 s and 360 s would be required to reach 3 log-inactivation of *L. monocytogenes* in cooked and dry-cured ham, respectively.

Correlation between pressure-resistance and the characteristics of the strains

The geno- and phenotypical characterization of the fifteen strains of *L. monocytogenes* and the strain of *L. innocua,* and the cluster membership in both meat matrices is shown in **Table 1**. Our results suggested no correlation between pressure resistance and lineage, serogroup, some virulence factors, previous listeriosis-case history or the origin in cooked ham as well as in cured ham ($p > 0.05$).

Lineage I strains, which are often isolated from human clinical cases (Orsi et al., 2011) presented a wide range in resistance, being found the three clusters of both meat products. Similarly, serogroup 4 strains, which contains serotype 4b the most listeriosis-associated serotype, exhibited a variable tolerance. There are no previous studies that relates geno- or serotype and pressure resistance in *L. monocytogenes.* In other foodborne pathogens such as *E. coli*, there is no correlation between inactivation by high hydrostatic pressure and genotype or serotype (Liu et al., 2015). Geno- and serotyping have demonstrated to be poor predictors of *L. monocytogenes* growth, including similar conditions to those found in meat products (Arguedas-Villa et al., 2010; Begot et al., 1997; Pal et al., 2008). In contrast, the tolerance to salt stress seems to be related to lineage and serotype (Bergholz et al., 2010; Van Der Veen et al., 2008). Although the origin did not show a strong correlation with pressure-resistance ($p > 0.05$), human-isolated strains seemed to be more sensitive than meat-isolated ones, since most of them were classified in cluster Ch-A. In contrast, all strains in cluster Ch-C, the most resistant were isolated from meat or meat products. In dry-cured meat, strains from both origins were homogeneously distributed in the three clusters. Aryani et al. (2015) found no correlation between heat resistance and origin of *L. monocytogenes* strains. Regarding the virulence of the strains, our findings suggested no relationship between

virulence factors or listeriosis-case history and their pressure resistance. In the case of *E. coli*, the studies of Garcia-Hernandez et al. (2015) and Sheen et al. (2015) suggest a similar inactivation patterns of virulent and non-virulent strains.

It is remarkable that the number and the membership of strains in the different clusters in cooked and dry-cured ham were different (**Table 1**). For instance, the most resistant clusters, cluster Ch-C consisted in two strains of *L. monocytogenes* (ILSI 29 and ITA 83) and the strain of *L. innocua,* whereas its analogous in dry-cured ham, cluster Dh-A was constituted by five *L. monocytogenes* strains (including ILSI 29) and *L. innocua* strain. Our results suggested that pressure-resistance variation at strain level depended on the food matrix, as well. Loading plot (**Figure 7A**) from principal component analysis allowed the visualization of this fact. The orthogonality between inactivation data in cooked ham (CH) from the three HPP conditions and the data from dry-cured ham implied linear independency, which means the inactivation level of each strain in cooked ham did not depend on the levels reached in dry-cured ham and viceversa. The difference in a_w between both meat products could explain this outcome. Low water activity contributes to stabilizing proteins against pressure-induced-denaturation (Georget et al., 2015). Besides protein stabilization, the exposure to moderate salt stress induces physiological and genetic changes, resulting in the adaptation to subsequent unrelated stresses in *Listeria monocytogenes* (Hill et al., 2002). This stress demonstrated to have a marked strain variability in the pathogen (Adrião et al., 2008). One of the genetic changes induced by salt stress is the expression of sigma factor σ^B. The activation of σ^B confers protection to a wide range of stress in *L. monocytogenes* by the redirection of expression of dependent genes associated to mechanisms for withstanding adverse conditions (Chaturongakul et al., 2008), including high hydrostatic pressure (Wemekamp-Kamphuis et al., 2004).

Table 1. Cluster membership of *Listeria monocytogenes* strains according to the pressure-induced inactivation in cooked and cured ham.

Strain[a]		Source of isolation[b]	Listeriosis-case history[c]	Lineage[d]	Serogroup[e]	Haemolysis activity[f]	Internalins genes[g]			Clustering	
Name	Other name						*inlA*	*inlC*	*inlJ*	Cooked ham	Dry-cured ham
ILSI 01	Scott A	Human	Epidemic	I	4	+	+	+	+	CH-A	DH-A
ILSI 07	FSL J1-177	Human	Sporadic	I	3	+	+	+	+	CH-B	DH-B
ILSI 11	FSL C1-056	Human	Sporadic	II	1	-	+	+	+	CH-A	DH-B
ILSI 13	FSL M1-004	Human	Sporadic	II	*Listeria* spp	-	+	+	+	CH-A	DH-A
ILSI 20	FSL J1-168	Human	Sporadic	III	*Listeria* spp	-	+	+	+	CH-A	DH-A
ILSI 29	FSL N3-013	Pâté	Epidemic	I	4	+	+	+	+	CH-C	DH-A
ILSI 33	FSL N3-031	Hot dog	Sporadic	II	1	+	+	+	+	CH-B	DH-B
ILSI 35	FSL R2-499	Human	Epidemic	II	1	-	+	+	+	CH-B	DH-C
ILSI 36	FSL N1-227	RTE meat	Epidemic	I	4	-	+	+	+	CH-B	DH-B
ILSI 38	FSL R2-501	Human	Epidemic	I	4	++	+	+	+	CH-B	DH-A
ILSI 41	FSL R2-763	Human	Epidemic	I	4	+	-	+	+	CH-B	DH-B
ILSI 42	FSL R2-764	Sliced deli meat	Epidemic	I	4	+	-	+	+	CH-B	DH-C
ITA 81	CECT 5366	Human	Sporadic	I	4	+	+	+	+	CH-A	DH-C
ITA 83	UdG 1010	Meat	Unknown	II	2	-	+	+	+	CH-C	DH-B
UBU 07		Chicken thigh	Unknown	I	4	+	+	+	+	CH-A	DH-B
L. innocua	UBU01	Meat	Unknown	N/A	*Listeria* spp	-	-	-	-	CH-C	DH-A

[a] International Life Science Institute, ILSI; Technical Agriculture Institute of Castilla y León, ITA; University of Burgos, UBU. Spanish Type Culture Collection, CECT.
[b,c] Information provided by the collection owners
[d] Information provided by the collection owners. * Classified according to its serogroup (Nadon et al., 2001)
[e] According to the method by Doumith et al. (2004)
[f] According to the CAMP test
[g] According to the method by Liu et al. (2007b)

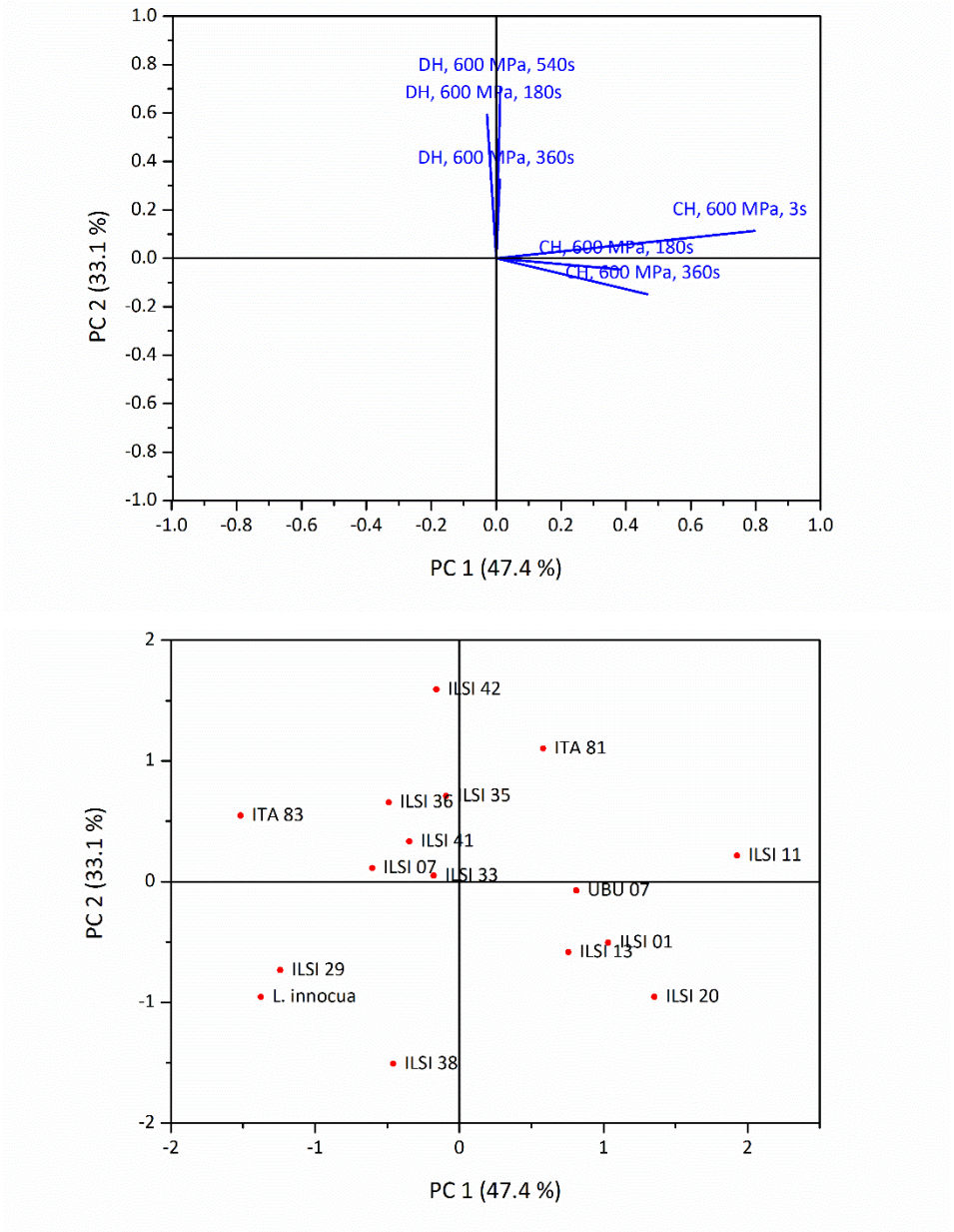


Figure 7. Principal component analysis loading (A) and score (B) plot of high hydrostatic pressure inactivation of *L. monocytogenes* strains (n = 15) and a strain of *L. innocua* in cooked (CH) and dry-cured ham (DH) based on covariance matrix.

Sigma factor σ^B regulates positively 168 genes of *L. monocytogenes* under salt stress (Raengpradub et al., 2008), thus it is possible that part of these regulated σ^B-dependent genes contributes to pressure resistance, as well. Strain variability could be related to a diverse stress-related gene expression within the same species, which could explain the different strain-to-strain resistance in the two tested meat matrices.

Proposal of *Listeria monocytogenes* cocktail for challenge tests of high pressure-processed meat products

Strain selection is an important decision when designing and conducting challenge tests for processing validation or optimization (Lianou and Sofos, 2007), including in challenge testing and validation of high pressure-processed food products (Balasubramaniam et al., 2004). Our results showed the existence of strains with higher pressure-resistance than average in both meat matrices (**Table 1**). We proposed five strain-cocktail for each meat product and a cocktail valid for both matrices (**Table 2**).

Table 2. *L. monocytogenes* strains included in the proposal cocktails and used as reference strains

Proposed cocktails			Other references	
Cooked ham cocktail	**Cured ham cocktail**	**Meat product cocktail**	**ILSI Human disease cocktail***	**Reference strains / Surrogate**
ILSI 29	ILSI 01	ILSI 13	ILSI 07	ILSI 01**
ILSI 33	ILSI 13	ILSI 29	ILSI 11	*L. innocua* UBU
ILSI 36	ILSI 20	ILSI 36	ILSI 29	
ILSI 42	ILSI 29	ILSI 38	ILSI 35	
ITA 83	ILSI 38	ITA 83	ILSI 36	

* (Fugett et al., 2006)
** Other denomination: Strain *Listeria monocytogenes* Scott A

Strain selection in each meat matrix was based on the recommendations from several authors (Balasubramaniam et al., 2004; Lianou and Koutsoumanis, 2013; NACMCF, 2010). In dry-cured ham, we selected those *L. monocytogenes* strains which exhibited higher pressure-tolerance (cluster Dh-A). This cocktail comprised strains from lineage I, II and III, isolated from human clinical cases and meat and the some strains have been related to listeriosis outbreaks. Strains in cooked ham cocktail came from cluster Ch-C (ILSI 29 and ITA 83), the most resistant group, whereas the other three (ILSI 33, ILSI 36 and ILSI 42) were selected because their intermediate resistance and other characteristics. ILSI 33 is serotype 1/2a, which was the most commonly human isolate in Europe during 2013 (EFSA, 2015), and ILSI 36 and ILSI 42 because these strains were

isolated from RTE meat products associated to listeriosis outbreaks. In addition, we proposed a third cocktail taking into account the inactivation data from both meat products. According to the principal component analysis (**Figure 7**), the strains ILSI 29 and ILSI 38 showed a higher pressure resistance in both matrices than the rest of evaluated strains, therefore they were included. ITA 83 and ILSI 13 presented a high pressure tolerance in cooked and cured ham, respectively. ILSI 36 exhibited an intermediate resistance in both matrices and their origin (RTE meat) made it a good candidate to be included. Using molecular methods, it is possible to trace each strain in a cocktail. While the strains ILSI 13 (serogroup *Listeria* spp.) and ITA 83 (serogroup 2) could be easily differentiated by PCR serogrouping, the rest of serogroup 4 strains could be identified by their pulsed field gel electrophoresis (PFGE)-type (PFGE-type 20, 8 and 35 for ILSI 29, ILSI 38 and ILSI 36, respectively) according to the data provided by Fugett et al. (2006).

For comparison purpose, the mean value of inactivation data from each strain included in the proposed cocktails (**Table 2**) was compared to other reference microorganisms and cocktails (**Figure 8**). *L. monocytogenes* Scott A (ILSI 01) was included in the comparison because its extended use as reference strain, including in studies about high pressure-processed meat products (Chung et al., 2005; Lucore et al., 2000; Morales et al., 2006). The "human disease cocktail" developed by ILSI NA was included, as well. This cocktail was designed to cover the genetic diversity of human disease-associated *L. monocytogenes,* including one representative from the epidemic clones and the main PFGE-types, ribotypes and serotypes associated with listeriosis (Fugett et al., 2006). Finally, *L. innocua* UBU was included in the comparison due to the consideration of the species as a surrogate of *L. monocytogenes* (Merialdi et al., 2015; Porto-Fett et al., 2010).

The cocktails and reference microorganisms presented a particular behavior at the different high pressure conditions in the two meat matrices ($p < 0.05$; **Figure 8**). The inactivation of meat product cocktail was statistically similar to the proposed cooked ham cocktail (**Figure 8A**) and cured ham cocktail (**Figure 8B**). This result was relevant because it would allow the evaluation of listericidal effect of high hydrostatic pressure

in different meat matrices with one cocktail. However, these results should be verified in further studies in meat products other than cooked and cured ham.

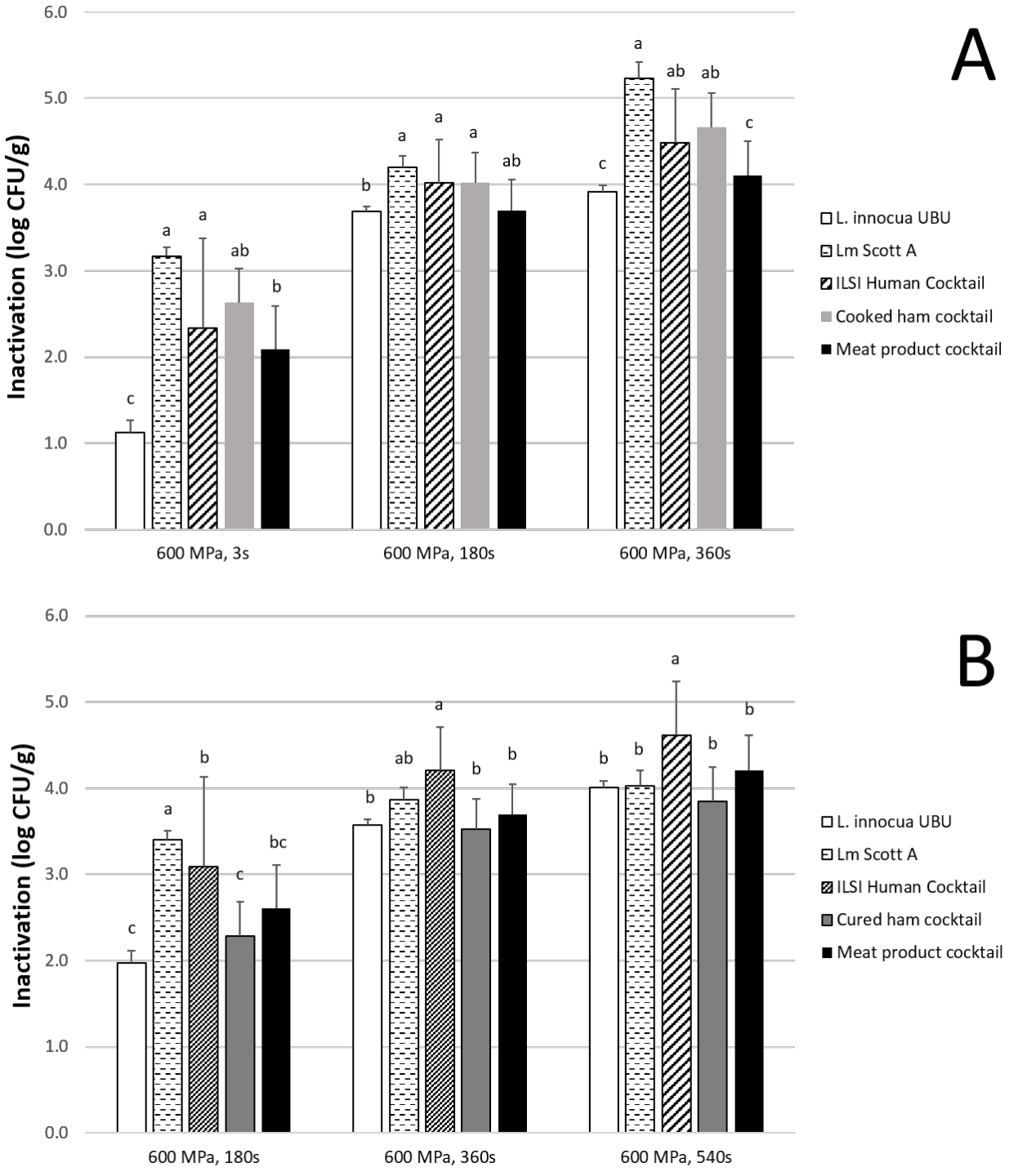


Figure 8. Effect of high pressure processing on inactivation of *L. monocytogenes* cocktails and reference strains in cooked ham (A) and dry-cured ham (B) after pressurization at 600 MPa. Data are represented as mean ± SD of the members of each cocktail or single strain. Different letters (a, b, c) at the same pressure and holding time indicate significant differences ($p < 0.05$) based on LSD test of significance.

According to our results, ILSI Human disease cocktail would be an adequate cocktail for using in challenge test of high pressure-processed-cooked ham, because it presented similar inactivation levels to cooked ham cocktail. However, its use would not be recommended in the assessment of cured ham due to its higher pressure-sensitive compared to the other *L. monocytogenes* cocktails proposed ($p < 0.05$).

The pressure-resistance of the Scott A (ILSI 01) strain was matrix-dependent. In dry-cured meat, the strain exhibited a high tolerance, whereas it was more sensitive to pressure in cooked ham, as we observed in cluster analysis (**Table 1**). Compared to the proposed cocktails, the strain exhibited lower pressure-resistance, being inactivated up to 1 log CFU/g more than meat product cocktail (**Figure 8A**). Our results were in agreement with the studies by Chung et al. (2005) and Morales et al. (2006), observing a high and low pressure tolerance in pressure-treated Serrano ham and sausages, respectively. This matrix dependency could discourage the use of Scott A as reference strain in all challenge tests and its suitability should be evaluated case-to-case.

Besides ILSI 29 strain, *L. innocua* UBU exhibited a higher pressure-resistance than the most of *L. monocytogenes* strains, regardless the meat matrix, being classified in cluster Ch-C and Dh-A (**Table 1**). The microorganism showed a higher tolerance among the proposed cocktails in cooked and cured ham, especially at short holding times ($p < 0.01$). The results obtained in this study suggested *L. innocua* UBU is a potential surrogate microorganism for *L. monocytogenes* in cooked and cured meat products, due to its nonpathogenic nature, reproducible growth, easy preparation, enumeration and stability (NACMCF, 2010).

Conclusions

L. monocytogenes showed differences in its pressure-tolerance at strain level. The strain variability depended on the processing conditions and the meat matrix. The strains were clustered in three groups for cooked ham and the same number for dry-cured ham

according to their inactivation levels. Holding time at 600 MPa affected in different way each cluster.

The number of pressure-resistant strains depended on the meat product. While two out of fifteen strains showed higher resistance than the average in cooked ham, five pressure-tolerant strains were detected in dry-cured meat. It could be due to a cross-protection phenomenon between salt stress and high hydrostatic pressure at physiological and genetic level. Pressure resistance showed no correlation with lineage, serogroup, virulence factors, pathogenicity or origin of the strains.

According to our results, current industrial conditions are adequate to control the presence of the pathogen in meat products. Processing cooked ham at 600 MPa for 180 s seems to be adequate conditions for reaching 3-log inactivation of the most resistant *L. monocytogenes* strains. Dry-cured ham would require 600 MPa during 360 s to achieve the same listericidal effect.

Although the meat matrix plays a relevant role in the strain variability of pressure-resistance, our study suggests that is possible to select five *L. monocytogenes* strains to constitute a cocktail for validation studies and challenge tests in diverse meat products. The *L. innocua* UBU strain exhibited a high pressure tolerance similar to the most of resistant strains of the pathogen in cooked and cured ham, suggesting to be a potential surrogate organism. These findings need further studies to be confirmed.

References

Adrião, A., et al. 2008. Marked intra-strain variation in response of *Listeria monocytogenes* dairy isolates to acid or salt stress and the effect of acid or salt adaptation on adherence to abiotic surfaces. *Int. J. Food Microbiol.* 123, 142–150.

Alpas, H., Kalchayanand, N., Bozoglu, F., Sikes, A., Dunne, C.P., Ray, B., 1999. Variation in resistance to hydrostatic pressure among strains of food-borne pathogens. *Appl. Environ. Microbiol*. 65, 4248–4251.

Arguedas-Villa, C., Stephan, R., Tasara, T., 2010. Evaluation of cold growth and related gene transcription responses associated with *Listeria monocytogenes* strains of different origins. *Food Microbiol*. 27, 653–660.

Aryani, D.C., den Besten, H.M.W., Hazeleger, W.C., Zwietering, M.H., 2015. Quantifying variability on thermal resistance of *Listeria monocytogenes*. *Int. J. Food Microbiol*. 193, 130–138.

Baer, A.A., Miller, M.J., Dilger, A.C., 2013. Pathogens of Interest to the Pork Industry: A Review of Research on Interventions to Assure Food Safety. *Compr. Rev. Food Sci. Food Saf*. 12, 183–217.

Balasubramaniam, V.M., Ting, E.Y., Stewart, C.M., Robbins, J. a., 2004. Recommended laboratory practices for conducting high-pressure microbial inactivation experiments. *Innov. Food Sci. Emerg. Technol*. 5, 299–306.

Begot, C., Lebert, I., Lebert, A., 1997. Variability of the response of 66 *Listeria monocytogenes* and *Listeria innocua* strains to different growth conditions. *Food Microbiol.* 14, 403–412.

Benito, A., Ventoura, G., Casadei, M., Robinson, T., Mackey, B., 1999. Variation in resistance of natural isolates of *Escherichia coli* O157 to high hydrostatic pressure, mild heat, and other stresses. *Appl. Environ. Microbiol*. 65, 1564–1569.

Bergholz, T.M., den Bakker, H.C., Fortes, E.D., Boor, K.J., Wiedmann, M., 2010. Salt stress phenotypes in *Listeria monocytogenes* vary by genetic lineage and temperature. *Foodborne Pathog. Dis.* 7, 1537–1549.

Betts, G., 2010. Challenge testing protocols for assessing the safety and quality of food and drink. Campden BRI Pubblications.

Bourdichon, F., 2014. *Listeria monocytogenes*: what have we learned from the last 30 years? *New food Mag. Food Saf. Suppl*. 17, 3–7.

Bover-Cid, S., Belletti, N., Aymerich, T., Garriga, M., 2015. Modeling the protective effect of aw and fat content on the high pressure resistance of *Listeria monocytogenes* in dry-cured ham. *Food Res. Int*. 75, 194–199.

Bover-Cid, S., Belletti, N., Garriga, M., Aymerich, T., 2011. Model for *Listeria monocytogenes* inactivation on dry-cured ham by high hydrostatic pressure processing. *Food Microbiol*. 28, 804–809.

Campus, M., 2010. High Pressure Processing of Meat, Meat Products and Seafood. *Food Eng. Rev*. 2, 256–273

Chaturongakul, S., Raengpradub, S., Wiedmann, M., Boor, K.J., 2008. Modulation of stress and virulence in *Listeria monocytogenes*. *Trends Microbiol.* 16, 388–396.

Chen, H., Neetoo, H., Ye, M., Joerger, R.D., 2009. Differences in pressure tolerance of *Listeria monocytogenes* strains are not correlated with other stress tolerances and are not based on differences in *CtsR*. Food Microbiol. 26, 404–408.

Chung, Y.K., Vurma, M., Turek, E.J., Chism, G.W., Yousef, A.E., 2005. Inactivation of barotolerant *Listeria monocytogenes* in sausage by combination of high-pressure processing and food-grade additives. *J. Food Prot*. 68, 744–750.

Cruz, J., 2013. Los productores de jamón curado buscan suplir con las exportaciones la caída de las ventas en España. *Eurocarne* 35–44.

de Noordhout, C.M., Devleesschauwer, B., Angulo, F.J., Verbeke, G., Haagsma, J., Kirk, M., Havelaar, A., Speybroeck, N., 2014. The global burden of listeriosis: a systematic review and meta-analysis. *Lancet Infect. Dis*. 14, 1073–1082.

Doumith, M., Buchrieser, C., Glaser, P., Jacquet, C., Martin, P., 2004. Differentiation of the Major *Listeria monocytogenes* Serovars by Multiplex PCR. *J. Clin. Microbiol*. 42, 3819–3822.

EFSA, ECDC, 2015. Trends and sources of zoonoses, zoonotic agents and food-borne. *EFSA J.* 13, 1–162.

FAO, 2004. Risk assessment of *Listeria monocytogenes* in ready-to-eat foods: Technical report, Microbiology risk assesment series 5. Geneva, Switzerland.

FSAI, 2005. The Control and Management of *Listeria monocytogenes* Contamination of Food. Dublin.

FSIS, 2014. FSIS Compilance Guideline: Controlling *Listeria Monocytogenes* in Post-lethaly Exposed Ready-to-Eat Meat and Poultry Products.

Fugett, E., Fortes, E., Nnoka, C., 2006. *Listeria monocytogenes* Strain Collection : Development of Standard *Listeria monocytogenes* Strain Sets for Research and Validation Studies. *J. Food Prot*. 69, 2929–2938.

Garcia-Hernandez, R., Mcmullen, L., Gänzle, M.G., 2015. Development and validation of a surrogate strain cocktail to evaluate bactericidal effects of pressure on verotoxigenic *Escherichia coli. Int. J. Food Microbiol*. 205, 16–22.

Garriga, M., Grèbol, N., Aymerich, M.T., Monfort, J.M., Hugas, M., 2004. Microbial inactivation after high-pressure processing at 600 MPa in commercial meat products over its shelf life. *Innov. Food Sci. Emerg. Technol*. 5, 451–457.

Georget, E., Sevenich, R., Reineke, K., Mathys, A., Heinz, V., Callanan, M., Rauh, C., Knorr, D., 2015. Inactivation of microorganisms by high isostatic pressure processing in complex matrices : A review. *Innov. Food Sci. Emerg. Technol*. 27, 1–14.

Haagsma, J., van der Zanden, B.P., Tariq, L., van Pelt, W., van Duynhoven, Y.T.H.P., Havelaar, A.H., 2009. Disease burden and costs of selected foodborne pathogens in the Netherlands, 2006. Bilthoven, The Netherlands.

Health Canada, 2010. *Listeria monocytogenes* Challenge Testing of Refrigerated Ready-to-Eat Foods. *Food Directorate*, Canada.

Health Canada, 2006. Use of High Hydrostatic Pressure for the Control of L. monocytogenes in Ready to Eat (RTE) Meats and Poultry. *Nov. Food Inf*. URL http://www.hc-sc.gc.ca/fn-an/gmf-agm/appro/nf-an138decdoc-eng.php (accessed 8.20.15).

Hereu, A., Bover-Cid, S., Garriga, M., Aymerich, T., 2012. High hydrostatic pressure and biopreservation of dry-cured ham to meet the Food Safety Objectives for *Listeria monocytogenes*. *Int. J. Food Microbiol*. 154, 107–112.

Hereu, A., Dalgaard, P., Garriga, M., Aymerich, T., Bover-Cid, S., 2012. Modeling the high pressure inactivation kinetics of *Listeria monocytogenes* on RTE cooked meat products. *Innov. Food Sci. Emerg. Technol*. 16, 305–315.

Hill, C., Cotter, P.D., Sleator, R.D., Gahan, C.G., 2002. Bacterial stress response *in Listeria monocytogenes*: jumping the hurdles imposed by minimal processing. *Int. Dairy J*. 12, 273–283.

ILSI, 2005. Achieving continuous improvement in reductions in foodborne listeriosis--a risk-based approach. *J. Food Prot.* 68, 1932–1994.

Jiang, J., Xiong, Y.L., 2014. Technologies and Mechanisms for Safety Control of Ready-to-Eat Muscle Foods: An Updated Review. *Crit. Rev. Food Sci. Nutr*. 55:13, 1886-1901.

Jofré, A., Aymerich, T., Grèbol, N., Garriga, M., 2009. Efficiency of high hydrostatic pressure at 600 MPa against food-borne microorganisms by challenge tests on convenience meat products. *LWT - Food Sci. Technol*. 42, 924–928.

Karatzas, K., Zervos, A., Tassou, C.C., Mallidis, C.G., Humphrey, T.J., 2007. Piezotolerant small-colony variants with increased thermotolerance, antibiotic susceptibility, and low invasiveness in a clonal *Staphylococcus aureus* population. *Appl. Environ. Microbiol*. 73, 1873–1881.

Koseki, S., Mizuno, Y., Yamamoto, K., 2007. Predictive modelling of the recovery of *Listeria monocytogenes* on sliced cooked ham after high pressure processing. *Int. J. Food Microbiol.* 119, 300–307.

Koseki, S., Yamamoto, K., 2007. Water activity of bacterial suspension media unable to account for the baroprotective effect of solute concentration on the inactivation of *L. monocytogenes* by high hydrostatic pressure. *Int. J. Food Microbiol*. 115, 43–47.

Lianou, A., Koutsoumanis, K.P., 2013. Strain variability of the behavior of foodborne bacterial pathogens: A review. *Int. J. Food Microbiol*. 167, 310–321.

Lianou, A., Sofos, J.N., 2007. A review of the incidence and transmission of *L. monocytogenes* in RTE products in retail and food service environments. *J. Food Prot*. 70, 2172–2198.

Liu, D., Lawrence, M.L., Austin, F.W., Ainsworth, A.J., 2007. A multiplex PCR for species- and virulence-specific determination of *Listeria monocytogenes*. *J. Microbiol. Methods* 71, 133–40.

Liu, Y., Gill, A., Mcmullen, L., Gänzle, M.G., 2015. Variation in Heat and Pressure resistance of Verotoxigenic and Nontoxigenic *Escherichia coli. J. Food Prot*. 78, 111–120.

Lucore, L.A., Shellhammer, T.H., Yousef, A.E., 2000. Inactivation of *Listeria monocytogenes* Scott A on artificially contaminated frankfurters by high-pressure processing. *J. Food Prot*. 63, 662–664.

Martín, B., Perich, A., Gómez, D., Yangüela, J., Rodríguez, A., Garriga, M., Aymerich, T., 2014. Diversity and distribution of *Listeria monocytogenes* in meat processing plants*. Food Microbiol.* 44, 119–127.

Martínez-Rodriguez, A., Mackey, B.M., 2005. Factors affecting the pressure resistance of some *Campylobacter* species. *Lett. Appl. Microbiol.* 41, 321–326.

Merialdi, G., Ramini, M., Ravanetti, E., Gherri, G., Bonilauri, P., 2015. Reduction of *Listeria innocua* contamination in vacuum-packaged dry-cured Italian pork products after high hydrostatic pressure treatment. *Ital. J. Food Saf.* 4, 101–103.

Morales, P., Calzada, J., Nuñez, M., 2006. Effect of high-pressure treatment on the survival of *Listeria monocytogenes* Scott A in sliced vacuum-packaged Iberian and Serrano cured hams. *J. Food Prot*. 69, 2539–2543.

NACMCF, 2010. Parameters for determining inoculated pack/challenge study protocols. *J. Food Prot*. 73, 140–202.

Nadon, C. a., Woodward, D.L., Young, C., Rodgers, F.G., Wiedmann, M., 2001. Correlations between molecular subtyping and serotyping of *Listeria monocytogenes. J. Clin. Microbiol*. 39, 2704–2707.

NSW food authority, 2013. Survey of Listeria monocytogenes in sliced pre-packaged RTE meats. Australia.

Orsi, R.H., Bakker, H.C.D., Wiedmann, M., 2011. *Listeria monocytogenes* lineages: Genomics, evolution, ecology, and phenotypic characteristics. *Int. J. Med. Microbiol*. 301, 79–96.

Pal, A., Labuza, T.P., Diez-Gonzalez, F., 2008. Comparison of primary predictive models to study the growth of *Listeria monocytogenes* at low temperatures in liquid cultures and selection of fastest growing ribotypes in meat and turkey product slurries. *Food Microbiol*. 25, 460–470.

Porto-Fett, A.C.S., Call, J.E., Shoyer, B.E., Hill, D.E., Pshebniski, C., Cocoma, G.J., Luchansky, J.B., 2010. Evaluation of fermentation, drying, and/or high pressure processing on viability of *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella* spp., and *Trichinella spiralis* in raw pork and Genoa salami. *Int. J. Food Microbiol*. 140, 61–75.

Raengpradub, S., Wiedmann, M., Boor, K.J., 2008. Comparative analysis of the sigma B-dependent stress responses in *Listeria monocytogenes* and *Listeria innocua* strains exposed to selected stress conditions. *Appl. Environ. Microbiol*. 74, 158–71.

RASFF, 2015. RASFF Portal [WWW Document]. Rapid Alert Syst. Food Feed. URL https://webgate.ec.europa.eu/rasff-window/portal/ (accessed 10.17.15).

Rendueles, E., Omer, M.K., Alvseike, O., Alonso-Calleja, C., Capita, R., Prieto, M., 2011. Microbiological food safety assessment of high hydrostatic pressure processing: A review. *LWT - Food Sci. Technol*. 44, 1251–1260.

Sheen, S., Cassidy, J., Scullen, B., Sommers, C., 2015. Inactivation of a diverse set of Shiga toxin-producing *Escherichia coli* in ground beef by high pressure processing. *Food Microbiol*. 52, 84–87.

Simpson, R., Gilmour, A., 1997a. The resistance of *Listeria monocytogenes* to high hydrostatic pressure in foods. *Food Microbiol*. 14, 567–573.

Simpson, R., Gilmour, A., 1997b. The effect of high hydrostatic pressure on *Listeria monocytogenes* in phosphate-buffered saline and model food systems. *J. Appl. Microbiol*. 83, 181–188.

Stollewerk, K., Jofré, A., Comaposada, J., Arnau, J., Garriga, M., 2012. The effect of NaCl-free processing and high pressure on the fate of *Listeria monocytogenes* and *Salmonella* on sliced smoked dry-cured ham. *Meat Sci*. 90, 472–477.

Tay, A., Shellhammer, T.H., Yousef, A.E., Chism, G.W., 2003. Pressure death and tailing behavior of *Listeria monocytogenes* strains having different barotolerances. *J. Food Prot*. 66, 2057–2061.

USDA-FSIS, 2015. FSIS Compliance Guideline HACCP Systems Validation April 2015.

Van Boeijen, I.K.H., Chavaroche, A., Valderrama, W.B., Moezelaar, R., Zwietering, M.H., Abee, T., 2010. Population diversity of *Listeria monocytogenes* LO28: Phenotypic and genotypic characterization of variants resistant to high hydrostatic pressure. *Appl. Environ. Microbiol.* 76, 2225–2233.

Van Der Veen, S., Moezelaar, R., Abee, T., Wells-Bennik, M.H.J., 2008. The growth limits of a large number of *Listeria monocytogenes* strains at combinations of stresses show serotype- and niche-specific traits. *J. Appl. Microbiol*. 105, 1246–1258.

Waite-Cusic, J.G., Diono, B.H.S., Yousef, A.E., 2011. Screening for *Listeria monocytogenes* surrogate strains applicable to food processing by ultrahigh pressure and pulsed electric field. *J. Food Prot*. 74, 1655–61.

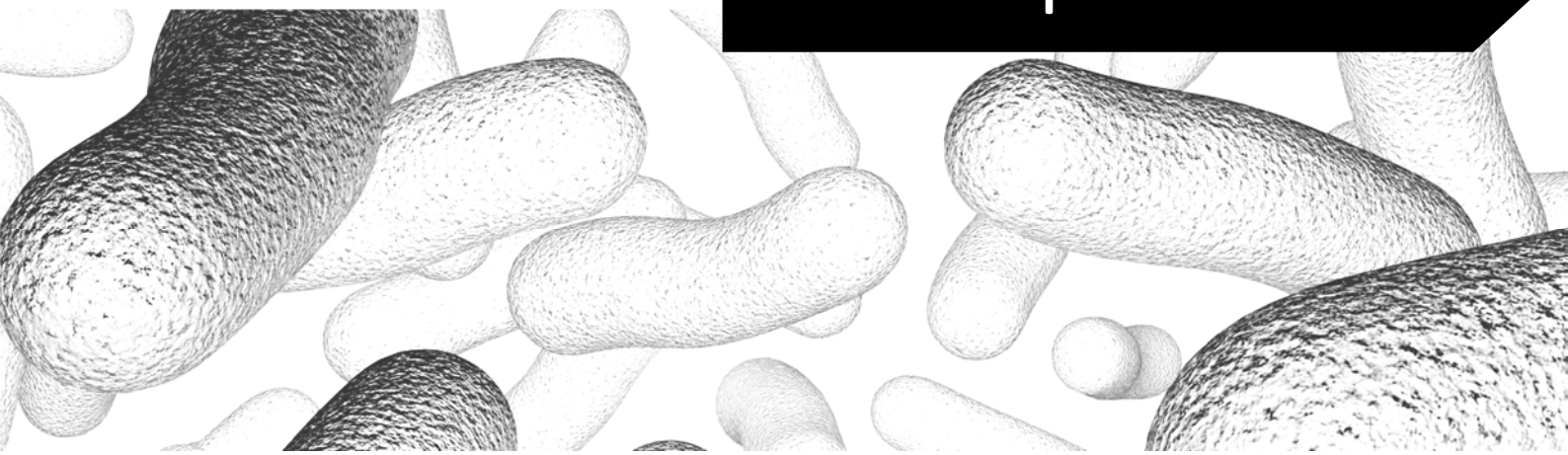
Wemekamp-Kamphuis, H.H., Wouters, J., Leeuw, P.P.L., De Hain, T., Chakraborty, T., Abee, T., 2004. Identification of sigma factor B -controlled genes and their impact on acid stress, high hydrostatic pressure, and freeze survival in *Listeria monocytogenes* EGD-e. *Appl. Environ. Microbiol.* 70, 3457–3466.

Whiting, R., Golden, M., 2002. Variation among *Escherichia coli* O157:H7 strains relative to their growth, survival, thermal inactivation, and toxin production in broth. *Int. J. Food Microbiol*. 75, 127–133.

Zhu, M., Du, M., Cordray, J., Ahn, D.U., 2005. Control of *Listeria monocytogenes* contamination in ready-to-eat meat products. *Compr. Rev. Food Sci. Food Saf*. 4, 34–42.

Validation of *Listeria innocua* as a surrogate of *Listeria monocytogenes* in high pressure-processed meat products

Chapter 3



Chapter 3

Validation of *L. innocua* as a surrogate of *L. monocytogenes* in high pressure-processed meat products

Introduction

Among bacterial-foodborne diseases, listeriosis has the highest hospitalization rate in European Union and in United States, reaching rates above 90 %. In addition, the disease causes by *L. monocytogenes* has had the highest case-fatality rate in both regions (15 % in EU and 19 % in US), according to food safety authorities (Crim *et al.*, 2014; EFSA and ECDC, 2015). The health-associated cost per case is considerably higher than other diseases caused by foodborne pathogens such as Shiga-toxin *Escherichia coli, Campylobacter* spp. and *Salmonella* spp. In The Netherlands, the cost-of-illness has reached € 106,922 per case (Mangen et al., 2015), while in United States, it has been estimated in USD 1.8 million per case (Hoffmann et al., 2015).

Besides the health-associated costs, the *L. monocytogenes* food contamination impacts on the economy of food industry. The cost of recalls caused by the presence of the pathogen is estimated between USD 1,200 and 2,400 million annually in USA (Ivanek et al., 2004). Foreign market access is limited by the presence of the pathogen, especially to these countries with a "zero-tolerance" policy about the presence of this microorganism in ready-to-eat (RTE) food products. Products such as dry-cured meats, highly appreciated RTE products are known 'vehicles' of *L. monocytogenes* (Doménech et al., 2015; Food Standards Australia- New Zealand, 2014; RASFF, 2015). Although they do not support the growth of the pathogen and have not been associated to listeriosis, its detection results problematic, limiting the exportation which has been estimated in € 237.5 million in Spain in 2012 (Cruz, 2013).

In order to achieve adequate levels of *L. monocytogenes* in the final product and meet the different regulations, hygiene rules should be followed strictly in food industries. In addition, post-lethally treatments or an antimicrobial agents or both that limit the growth of the pathogen can be used (USDA-FSIS, 2014). Different technologies can be used for controlling *Listeria monocytogenes* in RTE meat products such in-pack and surface pasteurization, irradiation, protective culture, addition of natural antimicrobials, and high pressure processing (Baer et al., 2013; Zhu et al., 2005).

According to FDA, high pressure processing (HPP) is a preservation technique that subjects previously packaged foods to pressures between 100 and 800 MPa and treatment times between 2 and 20 min (FDA, 2000). However, in current industrial HPP processing, the food products are processed from 400 to 600 MPa and holding times up to 10 min. This technology is currently used on an industrial scale in a wide range of products, including juices, dairy products, ready meals, fish and seafood or meat products. There is an increasing interest on the application of the technology on meat industry because it provides a high-added value and microbiological stability to these products (Bajovic et al., 2012).

Although the HPP technology can be used to guarantee the pathogen inactivation up to secure levels (Baer et al., 2013), some health authorities demand a science-based validation of the processing in any particular case (Health Canada, 2010; USDA-FSIS, 2014). The risk associated to use the pathogenic bacteria in in-plant testing, the need of trained people and specialized infrastructure for handling the pathogenic microorganism make interesting the use of surrogates for validation and challenge test studies. A surrogate can be defined as an organism or substance used to study the fate of a pathogen in a specific environment (Sinclair et al., 2012). The ideal surrogate is: nonpathogenic, similar inactivation kinetics to the target microorganism, stable growth characteristics, easily prepared to yield high-density populations, constant population until utilized, easily determined during analyses, susceptibility to injury similar to that of target microorganism and genetically stable (NACMCF, 2010). However, the search of surrogates must take into account the strain variability of the pathogen of concern, the food matrix and processing technology and its conditions. Recent studies have

demonstrated the existence of several non-pathogenic strains of *Escherichia coli* for use as surrogate of the pathogenic O157:H7, Verotoxigenic, and Shiga-toxin strains in high pressure processing studies in meat products (Garcia-Hernandez et al., 2015; Liu et al., 2015; Reineke et al., 2014).

Regarding surrogates for studies about *L. monocytogenes, L. innocua* is the most reasonable choice, because both microorganisms belong to the same taxonomic genus. Between the ten species within *Listeria* genus, *L. innocua* is genetically the closest species to *L. monocytogenes* (Den Bakker et al., 2010), and the two were not recognized as separate species until 1981 (Seeliger, 1981). Although the two species differ in pathogenicity, they share similar ecological niches in the environment, including meat products and meat processing surfaces (Guerra et al., 2001).

Despite the genetic similarity between species, comparative genomic studies have led to the discovery of 270 *L. monocytogenes* and 149 *L. innocua* species-specific genes (Glaser et al., 2001). Some of these genes are related to the ability of the both microorganisms to withstand stress conditions (Raengpradub et al., 2008), indicating the possibility of diverse response to environments and preservation technologies. According to the evidence, Milillo et al. (2012) concluded in their review that *L. innocua* is closely related to *L. monocytogenes*, significant genomic differences exist, though. The two species may have adapted to fit different environmental niches and do not always respond to stress the same way. Prior to field studies using *L. innocua* as a *Listeria monocytogenes* surrogate, appropriate preliminary studies confirming similar behavior of both species are necessary.

Both *Listeria* genus species have showed similar inactivation kinetics in other preservation technologies used in RTE meat products such as E-beam radiation and pulsed light (Hoz et al., 2008; Lasagabaster and de Marañón, 2012; Uesugi and Moraru, 2009). The use of *L. innocua* strains as surrogate in thermal processing of meat products has been demonstrated in several studies (Friedly et al., 2008; Kamat and Nair, 1996; Ma et al., 2007; Martínez-Rodriguez and Mackey, 2005; O'Bryan et al., 2006). In the case of HPP technology, there are no available data about the suitability of *L. Innocua* as surrogate in processing of meat products, though the microorganism has been used in

some studies (Carlez et al., 1993; Escriu and Mor-Mur, 2009; Merialdi et al., 2015; Vercammen et al., 2011). In dairy products, the results are inconclusive. Waite-Cusic et al. (2011) concluded that *Lactobacillus plantarum* ATCC 8014 is a more suitable surrogate microorganism of *L. monocytogenes* than *L. innocua* in pressure-treated sweet and acidified whey. However, both species presented a similar inactivation pattern in high pressure-processed yogurt drink (Evrendilek and Balasubramaniam, 2011). The latter stated the importance of the validation of the surrogate in each food matrix of interest.

Our previous studies showed that the strain *L. innocua* UBU presents a high tolerance to high hydrostatic pressure comparable to other pressure-resistant *L. monocytogenes* strains in a buffer system as well as in cooked and cured ham. Therefore, the main objective of this study was to evaluate this strain of *Listeria innocua* as a surrogate of *Listeria monocytogenes,* using a five pressure-resistant strain-cocktail, in high pressure processing of different meat matrices in conditions similar to those used at industrial level.

Materials and methods

Microorganisms

In the present study the strain *Listeria innocua* UBU was evaluated against a cocktail made of five strains of *Listeria monocytogenes*. The latter were selected due to demonstrated resistance to high pressure processing as previously stated in this PhD thesis. Their characterization is included in **Table 1**. *Listeria monocytogenes* strains were supplied by The International Life Science Institute – North America (ILSI, USA) and The Technical Agriculture Institute of Castilla y León (ITACyL, Spain). The strain of *L. innocua* belongs to University of Burgos strain collection (UBU).

Meat matrices

Meat matrices were chosen according to their expected physicochemical characteristics, and their association to *Listeria*-positive cases according to RASFF (2015) or their

economic impact for covering a wide range among meat products: Raw (minced beef), cooked (cooked chicken breast, cooked ham and fatty duck liver), cured (dry-cured ham and cured loin) and three types of fermented sausages (*chorizo*, salami and *salchichón*) were selected. Each product was purchased in a local supermarket, 24 h before the inoculation step and stored at 4 °C. Physicochemical analysis, pH and water activity (a_w), were done using a puncture pH-meter (CRISON, Spain) and water activity with Aqua-Lab (Decagon, US), respectively.

Table 1. Strains of *L. innocua* and *L. monocytogenes* used in this study and its genetic classification (lineage and serotype), origin and associated listeriosis cases.

Strain (Other name)[a]	**Lineage**[b]	**Serogroup**[c]	**Source of isolation**[d]	**Listeriosis-case history**[d]
L. innocua UBU	N/A	*Listeria* spp.	Meat product	Non-associated
L. monocytogenes strains				
ITA 083 (UdG 1010)	II*	1	Meat product	Non-associated
ILSI 13 (FSL M1-004)	II	*Listeria* spp.	Human	Sporadic case
ILSI 29 (FSL N3-013)	I	4	Pâté	Epidemic (UK, 1998-1990)
ILSI 36 (FSL N1-227)	I	4	RTE meat product	Epidemic (USA, 1998-1999)
ILSI 38 (FSL R2-501)	I	4	Human	Epidemic (USA, 2000)

[a] International Life Science Institute, ILSI; Technical Agriculture Institute of Castilla y León, ITA; University of Burgos, UBU.
[b] Information provided by the collection owners. * Classified according to its serogroup (Nadon et al., 2001)
[c] According to the method by Doumith et al. (2004)
[d] Information provided by the collection owners

Inoculum preparation

The stock cultures were kept frozen at −80 °C in 70 % brain heart infusion (BHI) broth (Oxoid, UK) and 30 % glycerol (v/v) (Sigma Aldrich, Spain). From the stock culture, a streak of each strain was made onto a tryptone soy agar and 0.6 % yeast extract (TSAYE, Oxoid, UK) plate and incubated for 24 h at 37 °C. A single colony was inoculated in a test tube pre-filled with 5 mL of BHI broth and incubated until the stationary phase for 18 h at 37 °C. Then, tubes were incubated at 37 °C during 24 h to obtain an initial population

of 10^9 CFU/mL. *L. monocytogenes* cocktail was prepared mixing 4 mL of each strain on a falcon tube to obtain a final mixture of 20 mL.

Inoculation

One-hundred microliters of *L. innocua* or *L. monocytogenes* cocktail inoculum were added and streaked on the surface of 10 g ± 0.5 g of each product, obtaining an initial population of approximately 10^7 CFU/g in the products and packaged in Stomacher bags (Interscience, France). Stomacher bags were put into another plastic bag and were vacuum packaged and kept under refrigeration overnight at 4 ^oC until high pressure treatment.

High pressure processing conditions

For all meat products, three holding times at 600 MPa were tested: 180, 360 and 540 s at room temperature (20 °C) in a 135-L high pressure unit (Hiperbaric 135 from Hiperbaric S. A., Burgos, Spain), using water as pressurization liquid. The pressure build-up time was 170 s for 600 MPa and the depressurization time was less than 3 s. The range of pressure and holding time was selected to cover usual industrial conditions. After high pressure treatment, the samples were stored at 4 °C until microbiological analysis (< 2 h).

Microbiological analysis

Ten-fold serial dilutions were made in Ringer's solution (Oxoid, UK) and depending on the expected counts the adequate decimal dilution was spread manually on Chromogenic Listeria Agar (OCLA; Oxoid, UK) and incubated at 37 °C for 48 h. The results were expressed in $\log_{10}$ colony-forming units (CFU)/g.

Data analysis

All inactivation results are given as the average ± standard deviation of triplicate samples and the two repetitions in non-consecutive days performed for each product (n = 6). Inactivation data was analyzed by means of variance analysis (one-way ANOVA) with the aim to establish statistical significance between data from two *Listeria* species in each meat product and HPP conditions. The statistical confidence level chosen was 95 %.

Finally, the suitability of *L. innocua* as surrogate of *L. monocytogenes* was assessed by means of linear regression, correlating inactivation values of both microorganisms. ANOVA and linear regression analyses were done using Statgraphics Centurion XVI (StatPoint Technologies, Inc., USA). All statistical procedures were done on inactivation data expressed in $\log_{10}$ colony-forming units (CFU)/g.

Results and discussion

Physicochemical characterization of meat matrices

The selected products covered a wide range of pH and a_w (**Figure 1**) from products with a low a_w such as cured and fermented sausages, to raw meat and cooked products with a_w above 0.95.

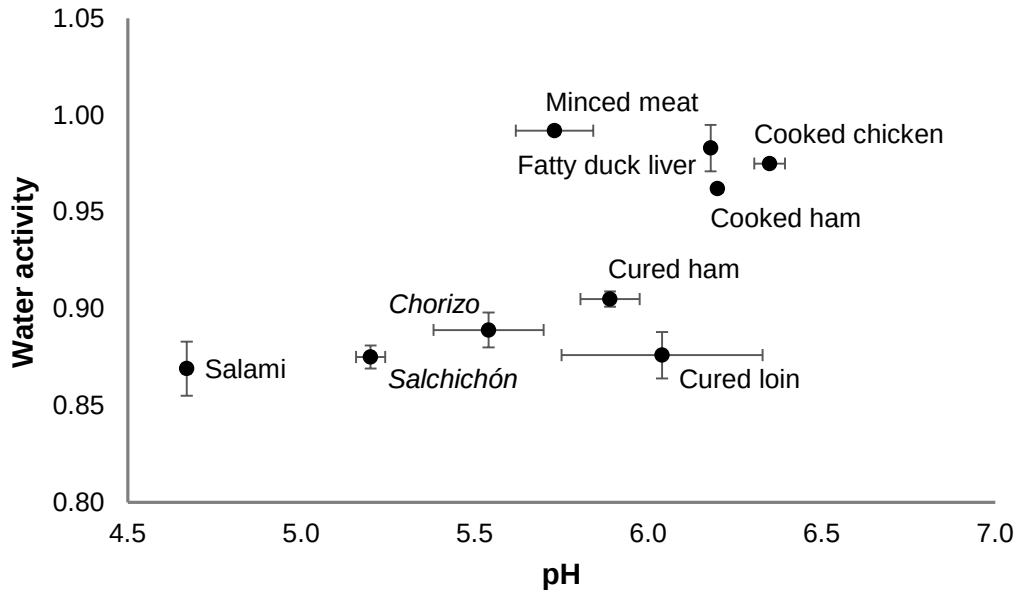


Figure 1. Physicochemical characterization (pH and a_w) of the nine meat matrices evaluated. Mean (point) and standard deviation (bars), n = 6.

Regarding the pH, meat products such as salami, *chorizo* and *salchichón*, which during their production have a fermentation step, presented the lower values of pH compared to cured, raw and cooked products. The range of these parameters for the different groups of meat products agreed with those reported by Toldrá (2007). Water activity

and pH are important variables in HPP processes since they are directly related with the degree of inactivation of microorganisms in food matrices (Alpas et al., 2000; Oxen and Knorr, 1993). In general, the lethal effect of high hydrostatic pressure on microorganisms is larger in food matrices at higher levels of a_w and lower pH values (Georget *et al.*, 2015).

Raw meat (minced beef)

High hydrostatic pressure (600 MPa during 180 s) was able to achieve above 4.5-log inactivation of both species of *Listeria* genus in minced beef (**Figure 2**). Increasing holding times at 600 MPa, from 180 s to 540 s, led to higher levels of inactivation up to 6 log cycles, approximately.

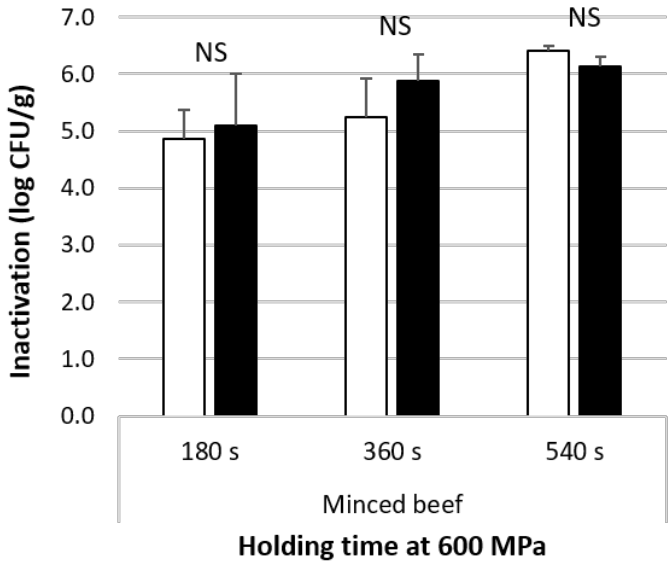


Figure 2. Inactivation of *L. innocua* UBU (white bars) and the five strains cocktail of *L. monocytogenes* (black bars) in minced beef by high pressure processing at 600 MPa for 180, 360 and 540 s. NS: $p > 0.05$; (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$

L. monocytogenes can be relatively easy-controlled by cooking procedures at 70 °C for a minimum of two minutes (Bourdichon, 2014). However, the tracking systems are still detecting the presence of the bacteria in these products. During 2014, RASFF system alerted about eight positive (below 100 CFU/g) *L. monocytogenes* cases in raw meat

(RASFF, 2015). Although the contamination level was low, the physicochemical characteristics of the meat product make it prone to support the growth of the pathogen. In agreement with our results, other studies demonstrated that the risk associated to the presence of the pathogen can be mitigated by high pressure processing in spite of the change in appearance induced by high hydrostatic pressure in raw meat products (Carlez et al., 1993; Castillo A. et al., 2004; Escriu and Mor-Mur, 2009; Jofré et al., 2009b; Kruk et al., 2011).

Comparing the inactivation levels between each species, we found no significant differences between them at all tested conditions ($p > 0.05$). According to our results, *L. innocua* UBU could be used as a surrogate organism for *L. monocytogenes* in this meat matrix. In similar meat matrices, both species had similar thermal inactivation patterns, suggesting the use of some strains of *L. innocua* as surrogate (Friedly et al., 2008; O'Bryan et al., 2006).

Cooked products (cooked ham, cooked chicken and fatty duck liver)

Inactivation levels for both species ranged from 3.5 to 5.8-log cycles at 600 MPa and up to 540 s in the evaluated cooked meat products: chicken, ham and fatty duck liver (**Figure 3**). The extension of holding time incremented the inactivation of both species. However, tripling the holding time increased less of 1 log-inactivation, regardless the bacteria or product processed.

The lethal effect varied between food matrices. At all holding times, the inactivation of both species was lower in cooked ham compared to the other cooked products (less than 1-log cycle). The slight differences in water activity between products (**Figure 1**) and the presence of other antimicrobials could partly explain the inactivation differences. It is known low a_w values exerts a baroprotective effect on bacterial inactivation (Koseki and Yamamoto, 2007). Preservatives such as sodium nitrate (E-250) are added in commercial formulations, included in these we tested. This salt exhibits a synergic effect with high hydrostatic pressure on inactivation of *L. monocytogenes* (De Alba et al., 2013).

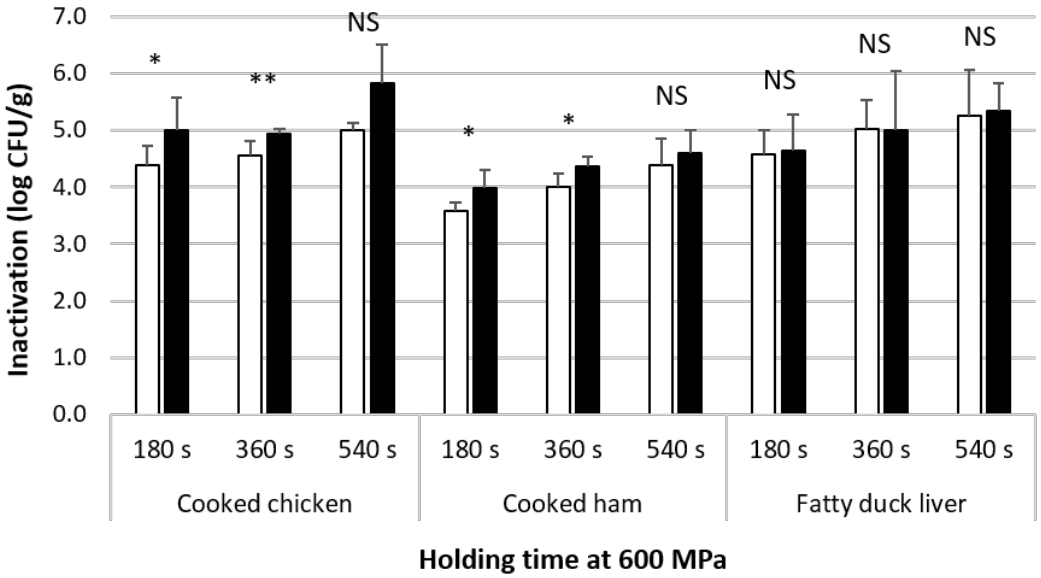


Figure 3. Inactivation of *L. innocua* UBU (white bars) and the five strains cocktail of *L. monocytogenes* (black bars) in three cooked meat products by high pressure processing at 600 MPa for 180, 360 and 540 s. NS: $p > 0.05$; (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$

In general, the inactivation pattern of *L. innocua* was similar to the cocktail of *Listeria monocytogenes* in pressure-treated cooked meat products. *L. innocua* was more pressure-resistant than the pathogenic bacteria ($p < 0.05$) in cooked chicken and ham at holding times below 360 s. Despite the statistical significance, the differences between both species were below 1 log CFU/g.

The inactivation levels of both species at 600 MPa were statistically equal ($p > 0.05$) in fatty duck liver at all holding times. Although high hydrostatic pressure is commercially used and has demonstrated to be effective for extending shelf-life of this meat product (Cruz et al., 2003), the information about the efficacy of HPP technology for controlling *L. monocytogenes* in this product is scarce. Belletti et al. (2011) achieved complete inactivation of *L. monocytogenes* on inoculated fatty duck liver with 100 CFU/g at 600 MPa.

L. monocytogenes was inactivated in a comparable range to other studies about the control of the pathogen in cooked poultry and ham (Garriga et al., 2004; Hereu et al.,

2012; Jofré et al., 2009b; Patterson et al., 2011). Our results were in agreement with those obtained by Jofré et al. (2009a). The authors reached a complete inactivation of the pathogen after processing cooked ham at 600 MPa during 360 s, starting an initial level of 3.5 log CFU/g of *L. monocytogenes*.

Ready-to-eat cooked meat products are well known vehicles of *L. monocytogenes,* and because of their physicochemical characteristics (pH > 6.0 , a_w > 0.95), the pathogen can grow causing the most important listeriosis outbreaks in Europe, North America and Oceania, with high hospitalization rate and deaths related to meat products (Bourdichon, 2014; NSW food authority, 2013). Since 2000, these products have been responsible of more than 100 of food alerts, 22 of them fatty duck liver and pâté-like cooked products have be involved in, and the same number for cooked ham (RASFF, 2015). According to our results, HPP technology is able to control both species of *Listeria* genus in cooked meat products, reaching levels from 3.5 up to 5.0 log-inactivation.

Inactivation levels obtained were high enough to validate HPP technology on RTE cooked meat products in countries such as United States or Canada, where levels of more than 2.0 log-inactivation or 3.0 log CFU/g of *L. monocytogenes* are required for process validation by food safety authorities (Health Canada, 2010; USDA-FSIS, 2014).

Cured products (Dry-cured ham and cured pork loin)

The lethal effect of HPP technology at 600 MPa on cured meat products ranged from 0.8 to 4.5 log CFU/g, depending on the food matrix and holding time (**Figure 4**). In dry-cured ham, the extension of holding time increased the inactivation levels in the cocktail of *L. monocytogenes* as well as *L. innocua* UBU, reaching up to 4 log-inactivation at 600 MPa for 540 s. In contrast, at the same conditions, both species were inactivated slightly above 2 log CFU/g in cured loin. The differences in a_w (0.905 ± 0.004 for cured ham and 0.876 ± 0.012 for cured loin) could explain the variation of lethal effect between cured meats. Bover-Cid et al. (2015) found 4 log-cycles of difference in inactivation of *Listeria monocytogenes* between dry-cured hams with a_w values of 0.860 and 0.960.

Despite the differences in the lethal effect between the cured products, the inactivation pattern at 600 MPa of *L. monocytogenes* was similar to the non-pathogenic species at all evaluated holding times. The latter suggests a potential use of *L. innocua* as surrogate in pressure-treated cured meats. Although there is no available comparative studies between both species in cured meat products, Merialdi et al. (2015) used successfully a five strains-cocktail of *L. innocua* to evaluate the efficacy of HPP on Italian dry-cured products. Similarly to cooked meat products, both species of *Listeria* genus have a comparable heat and radiation resistance in cured products (Hoz et al., 2008; Mackey et al., 1990).

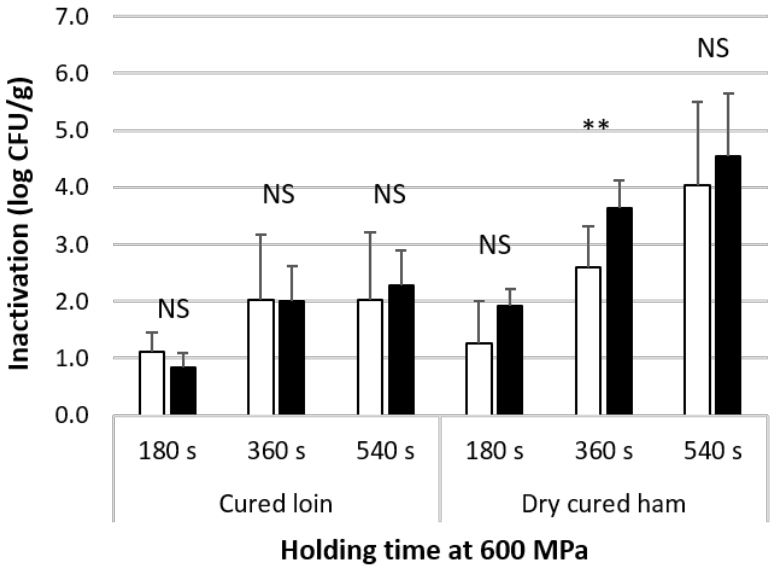


Figure 4. Inactivation of *L. innocua* UBU (white bars) and the five strains cocktail of *L. monocytogenes* (black bars) in two cured meat products by high pressure processing at 600 MPa for 180, 360 and 540 s. NS: $p > 0.05$; (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$

Although the curing and drying steps during manufacturing make this type of meat products microbiologically stable, *L. monocytogenes* has been isolated in cured meat products (Doménech *et al.*, 2015). These products have not been associated to listeriosis cases, however, the presence of the pathogen limits the access to those market demanding absence of the bacteria. RASFF have alerted eight times the presence of *L. monocytogenes* in cured meats (RASFF, 2015). During the period 2010 – 2012, Australian authorities blocked the entry of cured meat products from Italy and Spain around eight

times (NSW food authority, 2013). Thus, the need to control the pathogen in all RTE meat products to be exported.

Our results suggest the suitability of the technology for controlling *L. monocytogenes* in cured ham at 600 MPa for 540 s, achieving the inactivation levels demanded by food safety authorities (Health Canada, 2010; USDA-FSIS, 2014), in agreement with the reported results by Bover-Cid et al. (2015) and Jofré et al. (2009b). With regards to cured loin, it would be necessary the application of 600 MPa during 540 s for achieving 2-log inactivation of both microorganisms. The results agreed with those found by Corcuera et al. (2008), in which complete inactivation of *L. monocytogenes* was achieved after high pressure processing (600 MPa, 10 min) of samples of cured loin inoculated with the bacteria up to 3.4 log CFU/g.

Fermented products (Fermented sausages: *chorizo*, salami and *salchichón*)

Both, *L. innocua* and *L. monocytogenes* followed similar patterns in fermented meat products since *L. innocua* showed itself more resistant to HPP inactivation than the pathogen in all products and treatments evaluated (**Figure 5**).

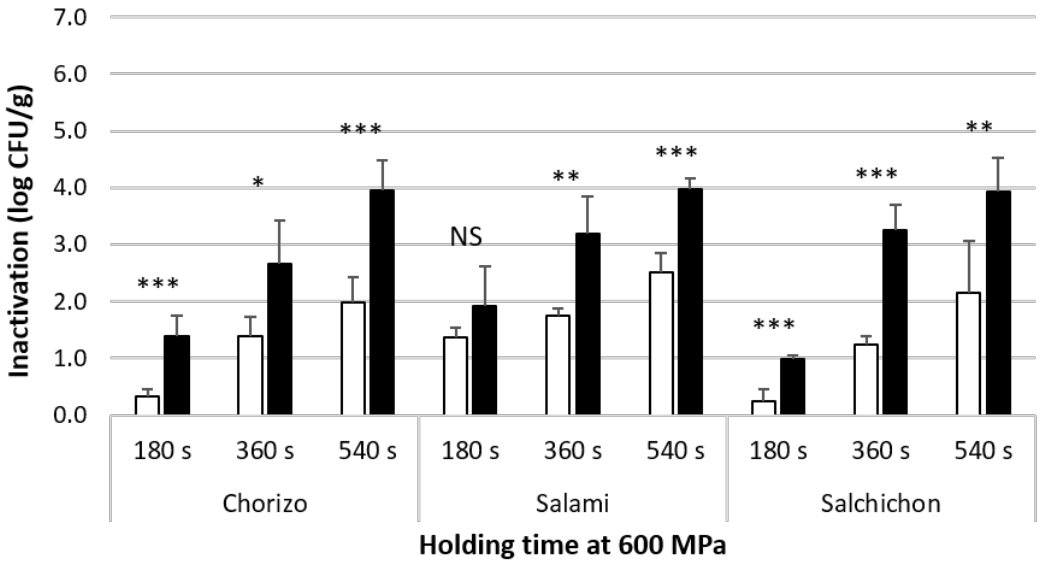


Figure 5. Inactivation of *L. innocua* UBU (white bars) and the five strains cocktail of *L. monocytogenes* (black bars) in three fermented meat products by high pressure processing at 600 MPa for 180, 360 and 540 s. NS: $p > 0.05$; (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$

In contrast with the observed in the other categories of meat products, inactivation levels achieved for both, *L. innocua* and *L. monocytogenes* were statistically different at the most of conditions ($p < 0.05$). *L. innocua* was around 1 log cycle more resistant than the cocktail of *L. monocytogenes* at all processing conditions in the three fermented products. Non-processed samples at 24 h after inoculation exhibited significant differences ($p < 0.05$) in salami (7.3 ± 0.2 log CFU/g and 6.6 ± 0.3 log CFU/g for *L. innocua* and *L. monocytogenes,* respectively). However, the counts in *chorizo* and *salchichón* were statistically equal ($p > 0.05$) around 7.0 log CFU/g for both microorganisms. Therefore, counts before HPP treatments explained partially the differences between species after high pressure processing caused by a lack of acid adaptation of one or more of the strains in the *L. monocytogenes* cocktail. Despite the genetic relatedness of both species, the genetic mechanisms involved in acid stress resistance differ between them (Raengpradub et al., 2008), explaining the variable adaptation to salami matrix. Several studies have demonstrated a higher inactivation of pressure-treated *L. monocytogenes* at lower pH than neutral (Alpas et al., 2000; Ritz et al., 2000). Different acid tolerance between species, in conjunction with a high pressure-low pH synergy could contribute to the disparity in inactivation levels of *Listeria* genus species. However, it was remarkable that *L. innocua* was more resistant than *L. monocytogenes,* hence, its use as surrogate is still suitable.

Similarly to cured products, there is no association between listeriosis cases and fermented meats, however, since 2000, 22 alerts have been notify for *Listeria monocytogenes*-positive cases (EFSA and ECDC, 2015; RASFF, 2015). Taking into account the data from *L. innocua* inactivation as the worst-case-scenario, HPP technology achieves the log-inactivation reductions required by food safety authorities (USDA-FSIS, 2014). However, 600 MPa during 540 s is necessary. At lower pressures and/or holding times, lethal effect would drop drastically, even below 1 log CFU/g (Jofré et al., 2009a). Inactivation level achieved for *L. monocytogenes* in salami agreed with these described by Porto-Fett et al. (2010), who obtained up to 5 log-inactivation of a five-strains cocktail in Genoa salami applying 600 MPa during 1 to 12 min.

Overall comparison between *L. innocua* and *L. monocytogenes* in diverse meat matrices

The evaluation of each type of meat products independently, showed a similar pattern of inactivation between *L. innocua* UBU and the cocktail of *L. monocytogenes.* On this basis, we decided to make an overall comparison between the pathogen and its potential surrogate. Inactivation was dependent on processing conditions and the characteristics of each meat product as it has been discussed in the previous sections. **Figure 6** shows the inactivation achieved for both microorganisms at the different treatments applied in each meat matrix, as a function of pH and a_w of each product. Inactivation levels are represented in bubbles, and their size is directly proportional to the inactivation level, showing the relevance of the characteristics of food matrix on the efficacy of microbial inactivation by high hydrostatic pressure. The overall evaluation allowed to visualize: (i) the high influence exerted by a_w on the lethal efficacy of HPP and (ii) the effect of pH was noticeable at shorter holding times.

For the global validation of the strain *L. innocua* UBU as a surrogate of *L. monocytogenes* in high pressure-processed meat products, inactivation values obtained for *L. innocua* (X-axis) were graphically represented against those obtained for the pathogen (Y-axis) and finally adjusted to a linear regression model (**Figure 7**). Inactivation values of all replicates performed (n = 6) for each product and treatment applied were included.

Statistical analysis of linear regression evidenced a significant linear relationship between inactivation of both species ($p < 0.05$). The purpose of the linear model was not to predict the inactivation on *L. monocytogenes* based on the results from *L. innocua* but getting information about the suitability of the non-pathogenic microorganism as surrogate in the different meat matrices and at variable conditions at 600 MPa. In linear model, the slope shows the proportion between inactivation levels of both species. A value of 1.0 is considered to be the ideal situation, indicating a perfect fit of lethal effect of HPP on both microorganisms. It would mean that 1 log-inactivation in the surrogate would correspond to 1 log-inactivation in the pathogen. At slope > 1.0, surrogate would have higher resistance than the pathogen. Although this is not the ideal situation, the

potential surrogate would be suitable for using in validation and challenge testing based on worst-case-scenario approach.

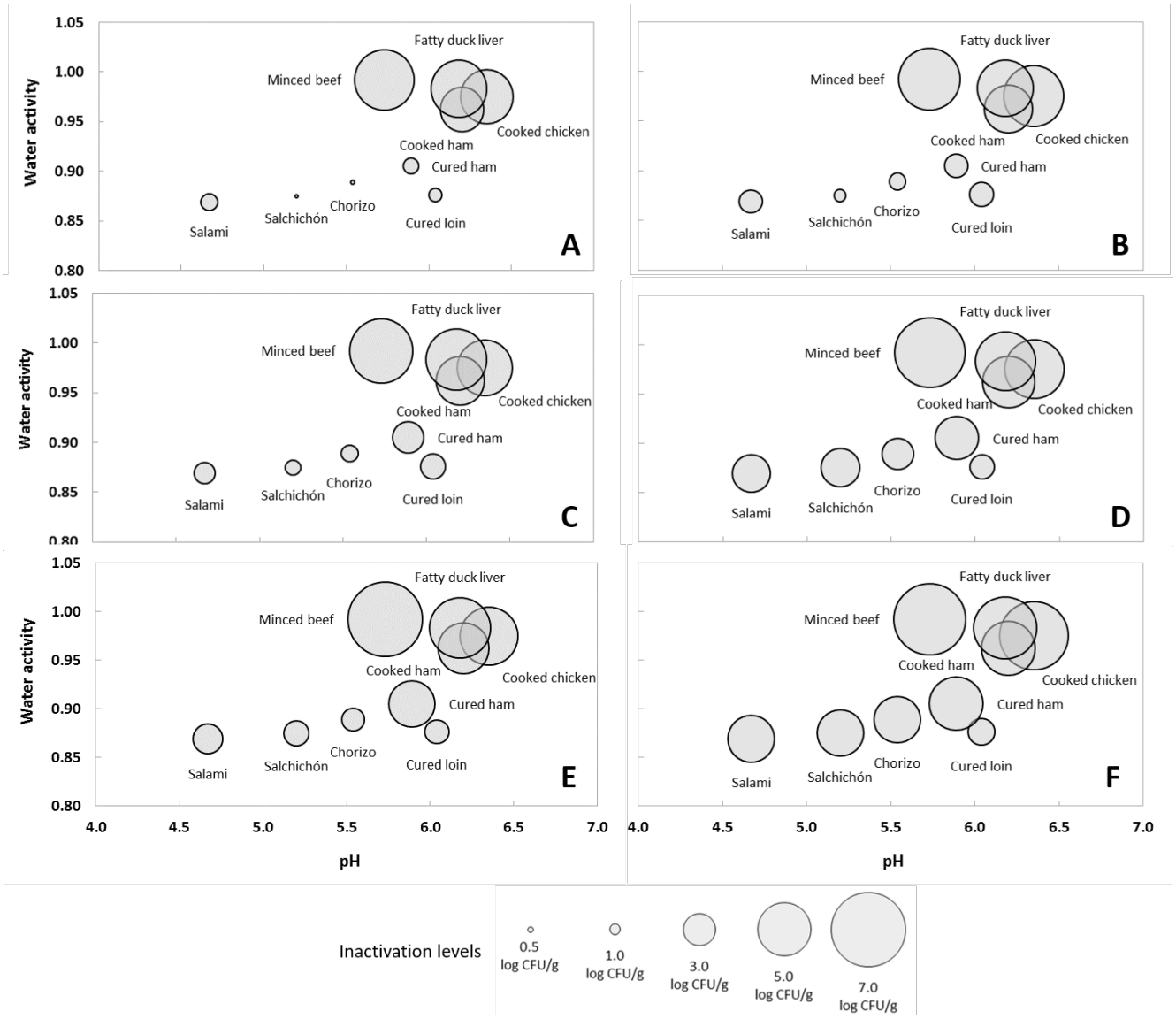


Figure 6. Inactivation levels of *L. innocua* (A, C, E) and the cocktail of five *L. monocytogenes* strains by high hydrostatic pressure at 600 MPa for 180 s (A, B), 360 s (C, D) and 540 s (E, F) according to the pH and water activity of the nine meat products selected.

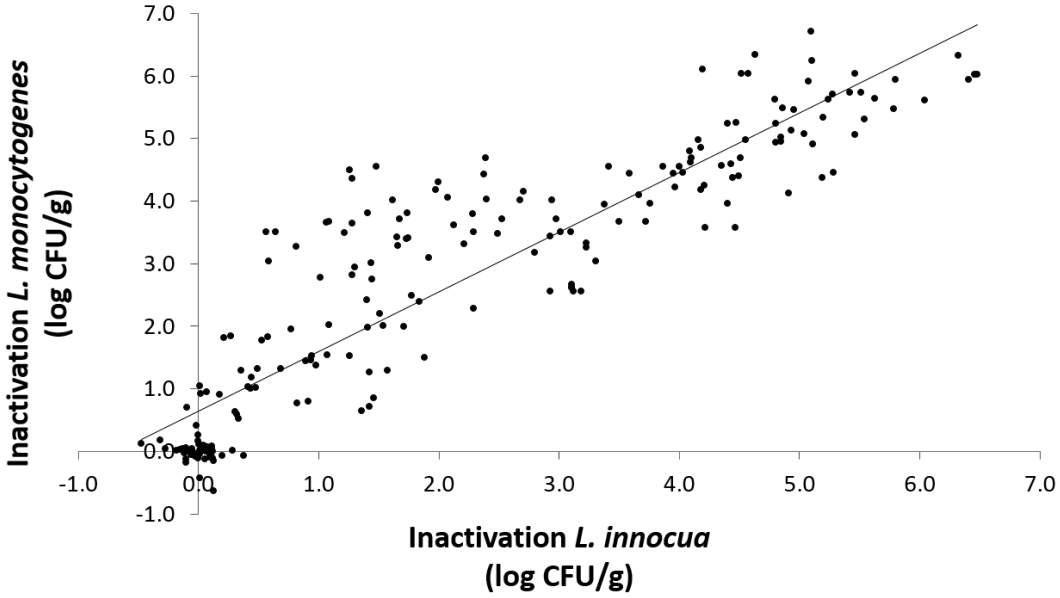


Figure 7. Dispersion plot of inactivation levels of the five-strain cocktail of *L. monocytogenes* against *L. innocua* for the nine meat products and high pressure processing conditions. Straight Line represents linear regression model y = 0.95x + 0.65 (R^2 = 0.826)

On the contrary, a slope below 1.0 would indicate the surrogate is more pressure-sensitive than the pathogen. In the latter case, the use of this surrogate would not be appropriate. Analogously to slope, Y-intercept is another parameter that shows how good the surrogate inactivation fits to the pathogen's one, especially at low inactivation values.

The equation obtained was $y = 0.95x + 0.65$ (R^2 = 0.826), which indicates that 82 % of variability is explained with the linear model selected for the adjustment of the data. The slope (0.953; $p < 0.05$) evidenced that the response of the strain *L. innocua* UBU to high hydrostatic pressure in meat products was similar to that of the cocktail of *L. monocytogenes* used in this study. With regards to the Y-intercept, it can be observed that it took a value of 0.65 ($p < 0.05$). The ideal situation would have been considered that in which the line was placed over the origin, taking the intercept a value of 0.0. The fact that the value of this parameter is greater than 0 indicates that at no inactivation of *L. innocua*, there is a slight inactivation of *L. monocytogenes* around 0.6 log CFU/g. This could be due to the differences in counts of both species in not-processed salami.

Linear regression model represented in **Figure 7** was elaborated including all inactivation values for both bacteria and for each product evaluated. **Table 2** includes the slope and Y-intercept values for the different types of meat products. Considering separately the slopes for each group of meat matrices, raw and cooked products had slope values close to one, which evidences that *L. innocua* and *L. monocytogenes* followed a similar inactivation pattern. With regards to cured products, the model parameters indicated a good fitting between both species, similar to those obtained in raw and cooked products. However, cured loin provided the lowest slope value of all products evaluated in this study. The slope calculated from inactivation data in fermented products are the highest. However, the use of *L. innocua* UBU strain as surrogate in validation and challenge testing is still advisable due to it higher pressure-tolerance to acidic conditions. On the other hand, the proposed cocktail should be modified for using in this kind of meat products.

Table 2. Linear regression model parameters (slope, Y-intercept and coefficient of determination) of inactivation data of *L. innocua* UBU and a five-strain cocktail of *L. monocytogenes* for each meat product evaluated

Category	Product	Slope $\left(\frac{\log \text{CFU } L. \textit{ monocytogenes}}{\log \text{CFU } L. \textit{ innocua}}\right)$	Y-intercept (log CFU/g)	R^2
Raw	Minced meat	0.98	0.39	0.914
Cooked	Cooked chicken	0.94	0.87	0.826
	Cooked ham	1.05	0.13	0.965
	Fatty duck liver	0.99	0.05	0.948
	Group mean	***0.98***	***0.39***	***0.897***
Cured	Cured loin	0.77	0.29	0.746
	Cured ham	0.97	0.62	0.823
	Group mean	***0.95***	***0.35***	***0.791***
Fermented	*Chorizo*	1.62	0.50	0.797
	Salchichón	1.32	0.85	0.593
	Salami	1.53	0.11	0.830
	Group mean	***1.42***	***0.57***	***0.712***
Overall meat products		**0.95**	**0.65**	**0.826**

Our results demonstrated similar patterns of inactivation of *L. innocua* compared to a cocktail of *L. monocytogenes* which exhibited high pressure-tolerance in diverse types of meat products at industrial conditions of HPP processes. Although *L. innocua* has been used as surrogate of *L. monocytogenes* in studies about high pressure-processed meat products (Carlez et al., 1993; Escriu and Mor-Mur, 2009; Merialdi et al., 2015; Vercammen et al., 2011), this is the first study that demonstrated its applicability as surrogate in a wide range of meat products and processing conditions. Other authors stated the suitability of *L. innocua* as surrogate in pressure-treated foods other than meat products, when they found no significant differences in kinetic inactivation of both *Listeria* genus species (Evrendilek and Balasubramaniam, 2011; Santillana-Farakos and Zwietering, 2011). In contrast, Tay et al. (2003) and Waite-Cusic et al. (2011) showed *Listeria innocua* ATCC 33090 has an intermediate pressure-resistance compared to *Listeria monocytogenes* and other potential surrogates evaluated.

L. innocua has traditionally been used as surrogate of *L. monocytogenes* in the development and validation of thermal processes in meat industry (Friedly et al., 2008; Kamat and Nair, 1996; Mackey et al., 1990; O'Bryan et al., 2006). However, it is not clear that the behavior of this microorganism is similar to that of the pathogen in all situations.

Preservation technology and food matrix play important roles for validation of *L. innocua* as surrogate. The control of *L. monocytogenes* by E-beam radiation was similar to the non-pathogenic *Listeria* species in dry-cured ham according to Hoz et al. (2008). However, the studies of Niemira (2010) and Rodriguez et al. (2006) found significant differences between both species in other matrices. Regarding natural antimicrobials, the suitability of *Listeria innocua* as surrogate depended on the evaluated compound. Under the exposure to carvacrol, both species presented different growth kinetics, whereas their growth was similar in media containing citral (Silva-Angulo et al., 2014, 2015).

Phylogenetic studies in *Listeria* genus suggest that *L. innocua* and *L. monocytogenes* (lineage IV) belong to sister groups because an ancient recombination event in *sigB* gene (Den Bakker et al., 2010). This gene plays a central role since it redirects gene expression, conferring protection to a wide range of stress in *L. monocytogenes* and other Gram-

positive bacteria (Chaturongakul et al., 2008; Wells-Bennik et al., 2008). Despite of the closeness between species, the differences in species-specific σ^B dependent genes are remarkable (Raengpradub et al., 2008). In *L. monocytogenes*, 168 genes were positively regulated by σ^B, while in *L. innocua*, 64 genes were positively regulated. The authors also found a common set of at least 49 genes that are σ^B-dependent in both species, which can contribute to explain the similarities and differences in resistance to several stresses, from a genomic standpoint.

Conclusions

The results obtained in the present study suggest *Listeria innocua* UBU is a valid surrogate of *Listeria monocytogenes* in meat matrices processed with high hydrostatic pressure. The strain follows a similar inactivation pattern to *Listeria monocytogenes* in raw, cooked and cured meat products processed at high pressure at 600 MPa for up to 540 s. Although both species exhibited different resistance in pressure-processed fermented meat products, the higher tolerance of *L. innocua* is still useful for validation and challenge studies based on the worst-case-scenario approach. Additionally, the latter suggests the need of improvement the proposed cocktail of pressure-resistant *L. monocytogenes* strains for using in future studies about these acidic products.

Although this work demonstrates the suitability of *L. innocua* as surrogate in high pressure-processed meat products, due to its not pathogenic behavior, similar inactivation characteristics, easily-laboratory handling and enumeration, the results cannot be extrapolated to other *L. innocua* strains and should be verified. Further research should be performed to find other adequate species or strains.

Water activity and pH play a relevant role in the inactivation of *L. monocytogenes* by high pressure technology and should be considered in the designing and optimization of processes for controlling the presence of the pathogen in meat industry.

Finally, it can be concluded that high pressure processing is a technology able to control the presence of *L. monocytogenes* in a variety of meat products. In addition, the strain

L. innocua UBU can be used in the validation and optimization of HPP processes in order to produce safer meat products when the absence of the pathogen must be guaranteed.

References

Alpas, H., Kalchayanand, N., Bozoglu, F., Ray, B., 2000. Interactions of high hydrostatic pressure, pressurization temperature and pH on death and injury of pressure-resistant and pressure-sensitive strains of foodborne pathogens. *Int. J. Food Microbiol.* 60, 33–42.

Baer, A., Miller, M.J., Dilger, A.C., 2013. Pathogens of interest to the pork industry: A review of research on interventions to assure food safety. *Compr. Rev. Food Sci. Food Saf*. 12, 183–217.

Bajovic, B., Bolumar, T., Heinz, V., 2012. Quality considerations with high pressure processing of fresh and value added meat products. *Meat Sci*. 92, 280–9.

Belletti, N., Martínez, B., Aymerich, T., Garriga, M., 2011. Inhibición *de Listeria monocytogenes* y *Salmonella* mediante alta presión hidrostática en un producto listo para el consumo como el bloc de foie gras. *Eurocarne* 197, 56–60.

Bourdichon, F., 2014. *Listeria monocytogenes*: what have we learned from the last 30 years? *New food Mag. Food Saf. Suppl*. 17, 3–7.

Bover-Cid, S., Belletti, N., Aymerich, T., Garriga, M., 2015. Modeling the protective effect of a_w and fat content on the high pressure resistance of *Listeria monocytogenes* in dry-cured ham. *Food Res. Int*. 75, 194–199.

Carlez, A., Rosec, J., Richard, N., Cheftel, J., 1993. High Pressure Inactivation of *Citrobacter freundii, Pseudomonas fluorescens* and *Listeria innocua* in Inoculated Minced Beef Muscle. *LWT - Food Sci. Technol.* 26, 357–363.

Castillo A., L.A., Mészáros, L., Kiss, I.F., 2004. Effect of high hydrostatic pressure and nisin on micro-organisms in minced meats. *Acta Aliment*. 33, 183–190.

Chaturongakul, S., Raengpradub, S., Wiedmann, M., Boor, K.J., 2008. Modulation of stress and virulence in *Listeria monocytogenes. Trends Microbiol*. 16, 388–396.

Corcuera, M.E., Díez Maté, A.M., Jaime Moreno, I., Tonello, C., 2008. Effectiveness of high hydrostatic pressure on detection of *L. monocytogenes* in pork cured loin by conventional microbiology and PCR real time analysis. *FoodMicro 2008*, Aberdeen, Scotland.

Crim, S.M., Iwamoto, M., Huang, J.Y., Griffin, P.M., Gilliss, D., Cronquist, A.B., Cartter, M., Tobin-D'Angelo, M., Blythe, D., Smith, K., Lathrop, S., Zansky, S., Cieslak, P.R., Dunn, J., Holt, K.G., Lance, S., Tauxe, R., Henao, O.L., 2014. Incidence and trends of infection with pathogens transmitted commonly through food- Foodborne Diseases Active Surveillance Network, 10 U.S. sites, 2006-2013. *MMWR. Morb. Mortal. Wkly. Rep*. 63, 328–32.

Cruz, C., El Moueffak, A., Antoine, M., Montury, M., Demazeau, G., Largeteau, A., Roy, B., Zuber, F., 2003. Preservation of fatty duck liver by high pressure treatment. *Int. J. Food Sci. Technol*. 38, 267–272.

Cruz, J., 2013. Los productores de jamón curado buscan suplir con las exportaciones la caída de las ventas en España. *Eurocarne* 35–44.

De Alba, M., Bravo, D., Medina, M., Park, S.F., Mackey, B.M., 2013. Combined effect of sodium nitrite with high-pressure treatments on the inactivation of *Escherichia coli* BW25113 and *Listeria monocytogenes* NCTC 11994. *Lett. Appl. Microbiol*. 56, 155–60.

Den Bakker, H.C., Bundrant, B.N., Fortes, E.D., Orsi, R.H., Wiedmann, M., 2010. A population genetics-based and phylogenetic approach to understanding the evolution of virulence in the genus *Listeria. Appl. Environ. Microbiol*. 76, 6085–6100.

Doménech, E., Jimenez -Belenguer, A., Amoros, J.A., Ferrus, M.A., Escriche, I., 2015. Prevalence and antimicrobial resistance of *Listeria monocytogenes* and *Salmonella* strains isolated in ready-to-eat foods in Eastern Spain. *Food Control* 47, 120–125.

EFSA, ECDC, 2015. Trends and sources of zoonoses , zoonotic agents and food-borne. *EFSA J*. 13, 1–162.

Escriu, R., Mor-Mur, M., 2009. Role of quantity and quality of fat in meat models inoculated with *Listeria innocua* or *Salmonella Typhimurium* treated by high pressure and refrigerated stored. *Food Microbiol*. 26, 834–40.

Evrendilek, G.A., Balasubramaniam, V.M., 2011. Inactivation of *Listeria monocytogenes* and *Listeria innocua* in yogurt drink applying combination of high pressure processing and mint essential oils. *Food Control* 22, 1435–1441.

FDA, 2000. Kinetics of microbial inactivation for alternative food processing technologies: High pressure procesing, *Safe Practices for Food Processes*.

Food Standards Australia- New Zealand, 2014. Food recall statistics [WWW Document].

Friedly, E.C., Crandall, P.G., Ricke, S., O'Bryan, C. a., Martin, E.M., Boyd, L.M., 2008. Identification of *Listeria innocua* surrogates for *Listeria monocytogenes* in hamburger patties. *J. Food Sci.* 73, 174–178.

Garcia-Hernandez, R., Mcmullen, L., Gänzle, M.G., 2015. Development and validation of a surrogate strain cocktail to evaluate bactericidal effects of pressure on verotoxigenic *Escherichia coli. Int. J. Food Microbiol*. 205, 16–22.

Garriga, M., Grèbol, N., Aymerich, M.T., Monfort, J.M., Hugas, M., 2004. Microbial inactivation after high-pressure processing at 600 MPa in commercial meat products over its shelf life. *Innov. Food Sci. Emerg. Technol.* 5, 451–457.

Georget, E., Sevenich, R., Reineke, K., Mathys, a, Heinz, V., Callanan, M., Rauh, C., Knorr, D., 2015. Inactivation of microorganisms by high isostatic pressure processing in complex matrices : A review. *Innov. Food Sci. Emerg. Technol*. 27, 1–14.

Glaser, P., et al., 2001. Comparative genomics of *Listeria* species. *Science* 294, 849–52.

Guerra, M.M., McLauchlin, J., Bernardo, F.A., 2001. *Listeria* in ready-to-eat and unprocessed foods produced in Portugal. *Food Microbiol*. 18, 423–429.

Health Canada, 2010. *Listeria monocytogenes* Challenge Testing of Refrigerated Ready-to-Eat Foods. *Food Directorate*, Canada.

Hereu, A., Dalgaard, P., Garriga, M., Aymerich, T., Bover-Cid, S., 2012. Modeling the high pressure inactivation kinetics of *Listeria monocytogenes* on RTE cooked meat products. *Innov. Food Sci. Emerg. Technol.* 16, 305–315.

Hoffmann, S., Maculloch, B., Batz, M., 2015. Economic Burden of Major Foodborne Illnesses Acquired in the United States. Washington D. C.

Hoz, L., Cambero, M.I., Cabeza, M.C., Herrero, A.M., Ordóñez, J.A., 2008. Elimination of *Listeria monocytogenes* from vacuum-packed dry-cured ham by E-beam radiation. *J. Food Prot*. 71, 2001–2006.

Ivanek, R., Gröhn, Y.T., Tauer, L.W., Wiedmann, M., 2004. The cost and benefit of *Listeria monocytogenes* food safety measures. *Crit. Rev. Food Sci. Nutr*. 44, 513–523.

Jofré, A., Aymerich, T., Garriga, M., 2009a. Improvement of the food safety of low acid fermented sausages by enterocins A and B and high pressure. *Food Control* 20, 179–184.

Jofré, A., Aymerich, T., Grèbol, N., Garriga, M., 2009b. Efficiency of high hydrostatic pressure at 600 MPa against food-borne microorganisms by challenge tests on convenience meat products. *LWT - Food Sci. Technol*. 42, 924–928.

Kamat, A., Nair, P.M., 1996. Identification of *Listeria innocua* as a Biological Indicator for Inactivation of *L. monocytogenes* by some Meat Processing Treatments. *LWT - Food Sci. Technol.* 720, 714–720.

Koseki, S., Yamamoto, K., 2007. Water activity of bacterial suspension media unable to account for the baroprotective effect of solute concentration on the inactivation of *Listeria monocytogenes* by high hydrostatic pressure. *Int. J. Food Microbiol*. 115, 43–47.

Kruk, Z.A., Yun, H., Rutley, D.L., Lee, E.J., Kim, Y.J., Jo, C., 2011. The effect of high pressure on microbial population, meat quality and sensory characteristics of chicken breast fillet. *Food Control* 22, 6–12.

Lasagabaster, A., de Marañón, I.M., 2012. Sensitivity to pulsed light technology of several spoilage and pathogenic bacteria isolated from fish products. *J. Food Prot.* 75, 2039–44.

Liu, Y., Gill, A., Mcmullen, L., Gänzle, M.G., 2015. Variation in Heat and Pressure resistance of Verotoxigenic and Nontoxigenic *Escherichia coli. J. Food Prot*. 78, 111–120.

Ma, L., Kornacki, J.L., Zhang, G., Lin, C.-M., Doyle, M.P., 2007. Development of thermal surrogate microorganisms in ground beef for in-plant critical control point validation studies. *J. Food Prot*. 70, 952–957.

Mackey, B.M., Pritchet, C., Norris, A., Mead, G.C., 1990. Heat resistance of *Listeria*: Strain differences and effects of meat type and curing salts. *Lett. Appl. Microbiol*. 10, 251–255.

Mangen, M.J., Bouwknegt, M., Friesema, I.H.M., Haagsma, J., Kortbeek, L.M., Tariq, L., Wilson, M., van Pelt, W., Havelaar, A.H., 2015. Cost-of-illness and disease burden of food-related pathogens in the Netherlands, 2011. *Int. J. Food Microbiol*. 196, 84–93.

Martínez-Rodriguez, A., Mackey, B.M., 2005. Factors affecting the pressure resistance of some *Campylobacter species*. *Lett. Appl. Microbiol*. 41, 321–326.

Merialdi, G., Ramini, M., Ravanetti, E., Gherri, G., Bonilauri, P., 2015. Reduction of *Listeria innocua* contamination in vacuum-packaged dry-cured Italian pork products after high hydrostatic pressure treatment. *Ital. J. Food Saf*. 4, 101–103.

Milillo, S.R., Friedly, E.C., Saldivar, J.C., Muthaiyan, A., O'Bryan, C., Crandall, P.G., Johnson, M.G., Ricke, S.C., 2012. A review of the ecology, genomics, and stress response of *Listeria innocua* and *Listeria monocytogenes. Crit. Rev. Food Sci. Nutr*. 52, 712–25.

NACMCF, 2010. Parameters for determining inoculated pack/challenge study protocols. *J. Food Prot.* 73, 140–202.

Niemira, B. a., 2010. Irradiation sensitivity of planktonic and biofilm-associated *Listeria monocytogenes* and *L. innocua* as influenced by temperature of biofilm formation. *Food Bioprocess Technol.* 3, 257–264.

NSW food authority, 2013. Survey of *Listeria monocytogenes* in sliced pre-packaged RTE meats. Australia.

O'Bryan, C., Crandall, P.G., Martin, E.M., Griffis, C.L., Johnson, M.G., 2006. Heat resistance of *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* 0157:H7, and *Listeria innocua* M1, a potential surrogate for *Listeria monocytogenes*, in meat and poultry: a review. *J. Food Sci*. 71, 23–30.

Oxen, P., Knorr, D., 1993. Baroprotective Effects of High Solute Concentrations against inactivation of *Rhodotorula rubra*. *LWT - Food Sci. Technol*. 26 (3), 220-223.

Patterson, M.F., Mackle, A., Linton, M., 2011. Effect of high pressure, in combination with antilisterial agents, on the growth of *Listeria monocytogenes* during extended storage of cooked chicken. *Food Microbiol*. 28, 1505–1508.

Porto-Fett, A.C.S., Call, J.E., Shoyer, B.E., Hill, D.E., Pshebniski, C., Cocoma, G.J., Luchansky, J.B., 2010. Evaluation of fermentation, drying, and/or high pressure processing on viability of *Listeria monocytogenes, Escherichia coli* O157:H7, *Salmonella* spp., and *Trichinella spiralis* in raw pork and Genoa salami*. Int. J. Food Microbiol*. 140, 61–75.

Raengpradub, S., Wiedmann, M., Boor, K.J., 2008. Comparative analysis of the sigma B-dependent stress responses in *Listeria monocytogenes* and *Listeria innocua* strains exposed to selected stress conditions. *Appl. Environ. Microbiol*. 74, 158–71.

RASFF, 2015. RASFF Portal [WWW Document]. Rapid Alert Syst. Food Feed. URL https://webgate.ec.europa.eu/rasff-window/portal/ (accessed 10.17.15).

Reineke, K., Sevenich, R., Hertwig, C., Janßen, T., Fröhling, A., Knorr, D., Wieler, L.H., Schlüter, O., 2014. Comparative study on the high pressure inactivation behavior of the Shiga toxin-producing *E. coli* O104:H4 and O157:H7 outbreak strains and a non-pathogenic surrogate. *Food Microbiol*. 46, 184–194.

Ritz, M., Jugiau, F., Rama, F., Courcoux, P., Semenou, M., Federighi, M., 2000. Inactivation of *Listeria monocytogenes* by high hydrostatic pressure : effects and interactions of treatment variables studied by analysis of variance. *Food Microbiol*. 17, 375–382.

Rodriguez, O., Castell-Perez, M.E., Ekpanyaskun, N., Moreira, R.G., Castillo, A., 2006. Surrogates for validation of electron beam irradiation of foods. *Int. J. Food Microbiol*. 110, 117–122.

Santillana-Farakos, S.M., Zwietering, M.H., 2011. Data analysis of the inactivation of foodborne microorganisms under high hydrostatic pressure to establish global kinetic parameters and influencing factors. *J. Food Prot*. 74, 2097–2106.

Seeliger, H.P.R., 1981. APATHOGENE LISTERIEN: *L. innocua* sp. *Zentralblatt fur Bakteriol*. *Mikrobiol. und Hyg.* - Abt. 1 Orig. A 249, 487–491.

Silva-Angulo, A.B., Zanini, S.F., Rodrigo, D., Rosenthal, A., Martinez, A., 2014. Growth kinetics of *Listeria innocua* and *Listeria monocytogenes* under exposure to carvacrol and the occurrence of sublethal damage. *Food Control* 37, 336–342.

Silva-Angulo, A.B., Zanini, S.F., Rosenthal, A., Rodrigo, D., Klein, G., Martínez, A., 2015. Comparative Study of the Effects of Citral on the Growth and Injury of *Listeria innocua* and *Listeria monocytogenes* Cells. *PLoS One* 10, e0114026.

Sinclair, R.G., Rose, J.B., Hashsham, S. a., Gerba, C.P., Haase, C.N., 2012. Criteria for selection of surrogates used to study the fate and control of pathogens in the environment. *Appl. Environ. Microbiol.* 78, 1969–1977.

Tay, A., Shellhammer, T.H., Yousef, A.E., Chism, G.W., 2003. Pressure death and tailing behavior of *Listeria monocytogenes* strains having different barotolerances. *J. Food Prot*. 66, 2057–2061.

Toldra, F., 2007. *Handbook of meat processing*, 1st ed. Wiley-Blackwell, Iowa.

Uesugi, A.R., Moraru, C.I., 2009. Reduction of *Listeria* on Ready-to-Eat Sausages after Exposure to a Combination of Pulsed Light and Nisin. *J. Food Prot*. 72, 347–353.

USDA-FSIS, 2014. FSIS Compliance Guideline : Controlling *Listeria monocytogenes* in Post-lethality Exposed Ready-to-Eat Meat and Poultry Products January 2014 143.

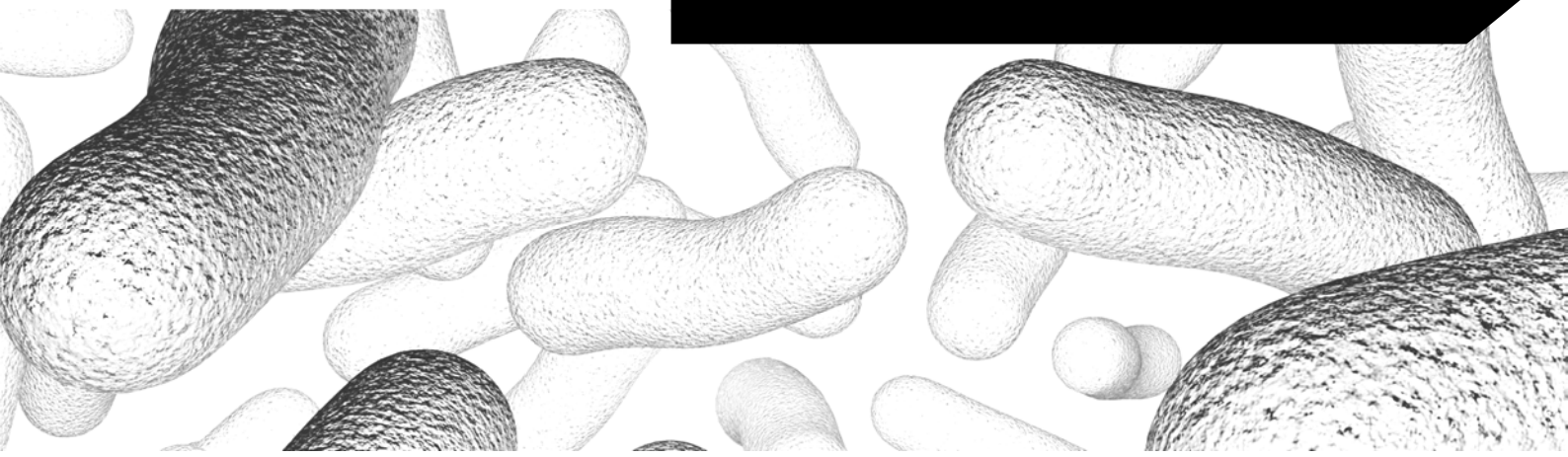
Vercammen, A., Vanoirbeek, K.G., Lurquin, I., Steen, L., Goemaere, O., Szczepaniak, S., Paelinck, H., Hendrickx, M.E.G., Michiels, C.W., 2011. Shelf-life extension of cooked ham model product by high hydrostatic pressure and natural preservatives. *Innov. Food Sci. Emerg. Technol*. 12, 407–415.

Waite-Cusic, J.G., Diono, B.H.S., Yousef, A.E., 2011. Screening for *Listeria monocytogenes* surrogate strains applicable to food processing by ultrahigh pressure and pulsed electric field. *J. Food Prot.* 74, 1655–61.

Wells-Bennik, M.H.J., Karatzas, K.A.G., Moezelaar, R., Abee, T., 2008. *Listeria monocytogenes*: High hydrostatic pressure resistance and survival strategies, in: Michiels, C., Bartlett, D., Aersten, A. (Eds.), *High-Pressure Microbiology*. ASM Press, Washington D. C., pp. 101–116.

Zhu, M., Du, M., Cordray, J., Ahn, D.U., 2005. Control of *Listeria monocytogenes* contamination in ready-to-eat meat products. *Compr. Rev. Food Sci. Food Saf*. 4, 34–42.

General discussion



General discussion

In the first part of this PhD thesis, we presented scientific evidence reported in the literature, showing that the differences at strain level have effect on how *Listeria monocytogenes* responds under the influence of diverse stresses. This work has focused on the study of high hydrostatic pressure, because of the current industrial use of this food preservation technology to control the pathogen in food products. *Listeria monocytogenes* strains seem to have a varied behavior under high pressure. However, the impact of strain variation on pressure-resistance of the pathogen, given the widespread diversity of the microorganism, remained unclear. Based on the state-of-the-art, we hypothesize that the resistance of *Listeria monocytogenes* to high pressure processing depends on each strain and the variation will be conditioned by the food matrix.

In **Chapter 1**, we evaluated the variation in inactivation and sublethal injury of thirty *L. monocytogenes* strains caused by high hydrostatic pressure in PBS buffer. Since the pathogen presents several geno- and phenotypic differences at strain level, the first part consisted in the characterization of 120 strains from one international collection, ILSI *Listeria* collection, one national collection, ITACyL collection, and our own strain collection (UBU). The purpose of including strains from national and international culture collections permits an intra-laboratory comparison and validation of the results achieved in our study, and to continue expanding the understanding of the mechanisms of *L. monocytogenes* for withstanding high hydrostatic pressure by using a standardized set of strains.

Following the characterization, we selected a sufficient number of strains for covering the natural diversity of the species in matters of evolutionary lineage, virulence and pathogenicity, and origin. Based on these criteria, we selected thirty out of the initial 120 strains for the next experiment about high pressure resistance.

Pressure resistance of *L. monocytogenes* was strain-dependent

These first results showed that high hydrostatic pressure provokes a lethal and sublethal effect in *L. monocytogenes* in PBS buffer (**Chapter 1**). The achieved levels of inactivation and sublethal injury depended on the strain as well as on the processing conditions. Ranges of inactivation and sublethal injury induced by high pressure were broader at intermediate conditions (400 MPa for 360 s, 500 MPa for 180 s and 600 MPa for 3 s). At the least and most intense conditions, high hydrostatic pressure exerted a similar lethal and sublethal effect regardless the strain.

According to the clustering analysis on inactivation data, the 30 strains could be classified in three major groups. One of the inactivation clusters, comprised of three strains, was significantly more pressure-resistant than the rest of strains, indicating that the most of *L. monocytogenes* strains can be classified to an intermediate-low pressure resistance. The use of PBS as a model system permitted to assess the lethal and sublethal effect on cells caused by high pressure, minimizing any synergetic or baroprotective effect of food components. However, the physicochemical characteristics of the solution (high water activity and neutral pH) can be found in few food products, limiting the scope of application in real food matrices. Therefore, the next step was to evaluate the pressure resistance of *L. monocytogenes* strains in RTE meat products (**Chapter 2**). Cooked and dry-cured ham were selected because their association with human listeriosis cases and the economic impact the presence of the pathogen entails on the export of these products.

Inactivation levels varied among the fifteen strains chosen for the study, at the different processing conditions in both meat matrices. Comparing the distributions of inactivation in cooked and dry-cured ham showed up the significant effect of food matrix in the variability of pressure resistance of *L. monocytogenes* strains. It was clearly observed a baroprotective effect exerted by the low a_w of dry-cured ham compared to inactivation in cooked ham at same pressure and holding time. In addition, inactivation in meat matrices was narrower than that achieved in PBS buffer, indicating that the synergetic/protective action induced by the food matrix is not global but this effect could vary at strain level, as well.

Cluster analysis on inactivation data in cooked ham, grouped the strains in three clusters, and an identical number of clusters were obtained in dry-cured ham. However, the number of members and the member strains were not the same. For instance, the number of *L. monocytogenes* strains in the most pressure-resistant cluster was lower in cooked ham (n = 3) than in dry-cured ham (n = 6), sharing only two members. In PBS buffer, the cluster C was comprised by three from a set of 30 strains.

Since the results suggested that food matrix exerted a significant effect on strain variability, we compared eight *L. monocytogenes* strains, which were present in strain set of buffer system, cooked ham and dry-cured ham. Strains ILSI 07, ILSI 11, ILSI 35 and ILSI 36 presented low/intermediate pressure-resistance in all matrices. Interestingly, only two strains, ILSI 29 and ITA 83, were clustered in high-resistant groups in PBS and meat products. The other two strains (ILSI 01 and ILSI 13) exhibited a similar tolerance to high hydrostatic pressure in PBS buffer and cooked ham, but distinct in dry-cured meat. Both strains were pressure-sensitive in buffer and cooked ham and were classified as resistant in dry-cured ham.

Our results showed that strain variability can be influenced by food matrix. Although the understanding of the mechanisms related to these phenomena were not covered by this work, the current knowledge on the modulation of stress in *L. monocytogenes* allowed us to hypothesize about how food matrix can influence the response to hydrostatic pressure at strain level as we expounded in **Chapter 2.**

Pressure resistance was not related to the evaluated geno- and phenotypical characteristics

Cluster analysis allowed grouping the *L. monocytogenes* strains according its resistance to high hydrostatic pressure in PBS buffer (**Chapter 1**) and ready-to-eat meat products (**Chapter 2**). Our results showed that there was no correlation between pressure-resistance and evolutionary lineage, serogroup, some virulence factors, origin or the previous listeriosis case history, regardless of the matrix. By contrast, these characteristics demonstrated a strong correlation with the *L. monocytogenes* strain variability resistance to other stresses, reported in the literature.

The interest in finding one or several characteristics that predict the resistance of *L. monocytogenes* is related to their use as "markers", in order to facilitate the selection of appropriate processing conditions based on the characteristics of the target strain. That task could be challenging due to complexity of genetic and protein systems involved in the stress resistance of the microorganism. Although the characteristics of the strains evaluated our results did not correlate with pressure tolerance, cluster membership is a valuable information in other to find geno- or phenotypical differences between pressure-resistant and sensitive strains. For the moment, challenge testing seems to be the most suitable and reliable approach for process validation.

Strain variability as a tool for designing safer HPP ready-to-eat meat products

It is known that the impact of strain variability on microbial behavior is significant. Previous studies in literature have demonstrated that the response of *L. monocytogenes* and other microorganisms to different stresses varies among the strains of a same species.

Our results showed that it was possible to select pressures and holding times, applicable to industrial conditions, in order to achieve the inactivation levels in *L. monocytogenes* requested for food safety authorities, regardless the strain. Inactivation levels above 5-log cycles were reached at 600 MPa during 360 s in PBS buffer (**Chapter 1**). Regarding meat products, processing at 600 MPa for 180 s and 360 s were necessary to inactivate the most pressure-resistant strains above 3 log cycles in cooked and dry-cured ham, respectively (**Chapter 2**). The selected conditions are in the range to those currently used by several meat companies with access to the HPP technology.

In academia and industry, some strains are often used, becoming in a reference for research or cocktails used in validation studies, however, their use is not always validated. *L. monocytogenes* Scott A, a milk-borne isolate with clinical importance, is one of these reference strains commonly used in research, including in studies about high hydrostatic pressure. Our findings suggested that the use of this strain (in our work was

named ILSI 01) in studies about high pressure technology should be limited. This strain exhibited higher resistance than the rest of set of *L. monocytogenes* only in dry-cured meat (**Chapter 2**). By contrast, its tolerance in PBS buffer (**Chapter 1**) and cooked ham was low. Although its use as reference strain is widespread in academia, the results could overestimate the lethality effect of HPP technology, given a false perception of safety, when it is used to evaluate the processing performance in high pressure-processed cooked ham. Therefore, we proposed ILSI 29 (a. k. a. FSL N3-013) to be a reference strain for high pressure-processed cooked and cured meat products, based on the results from this work. The strain exhibited a significant pressure-resistance in PBS as well as in meat products. It shares lineage, serogroup and virulence factors with Scott A. In addition, ILSI 29 was isolated from pâté linked to human listeriosis in United Kingdom in 1988 - 1989. These reasons make to ILSI 29 a good candidate to be a reference strain for testing HPP technology in RTE meat products.

From microbial risk assessment, the knowledge in strain variability provide a science-based information for a better selection of those strains to be included in a challenge test. The purpose of challenge testing is to validate the range of interventions/preservation technologies used to control food-borne pathogens. The analysis of strain variability permitted us to suggest five strains to be included in a cocktail for challenge testing (**Chapter 2**). The selected strains (ILSI 13, ILSI 29, ILSI 35, ILSI 42 and ITA 83) exhibited a high tolerance to high hydrostatic pressure in, at least, one of the two meat products. In addition, those strains were isolated from clinical human cases or meat products associated to listeriosis, following the recommendations for strain selection. The differences in geno- and phenotypical characteristics among them, makes possible tracing each one by using molecular methodologies such as PFGE and PCR serotyping in a mix of inoculums.

When the cocktail was challenged in nine meat products (raw, cooked, cured and fermented meats) at conditions used in industry (600 MPa for few minutes), it exhibited a good performance compared to a high pressure-tolerant *L. innocua* strain in raw, cooked and cured meat products evaluated (**Chapter 3**). However, the cocktail seemed to be sensitive to high pressure in fermented cured meat products. The latter suggested

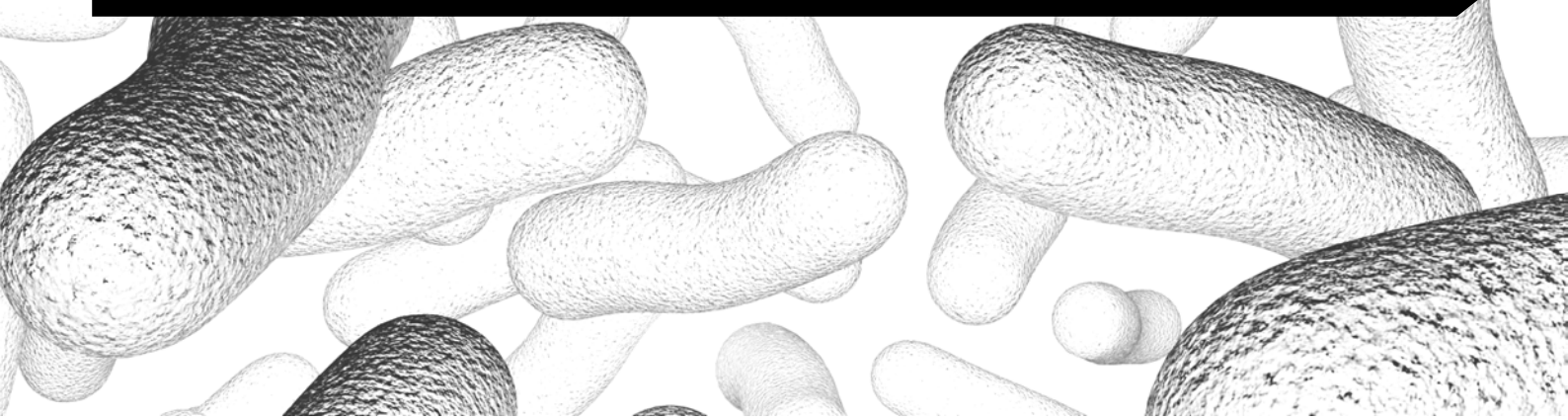
one or several strains in the cocktail are sensitive to low pH or the combination of low pH and low a_w values, limiting the application in those products.

L. innocua is a species closely related to *L. monocytogenes* from phylogenetic standpoint. Because of that, its use as surrogate of *L. monocytogenes* has been suggested for diverse preservation technologies. However, its suitability has been criticized because their genetic variation in stress response compared to *L. monocytogenes* which suggests the need of validation. Currently, the use as surrogate has been validated in thermal processing of meat products.

Regarding high hydrostatic pressure, it is assumed that the behavior of this bacterium is similar to the pathogenic relative, but it has not been validated. Originally, the strain of *L. innocua* UBU, isolated from a RTE meat product, was included in the first part of this work as negative control in the different analytical tests for strain characterization. However, this microorganism exhibited a high pressure-resistance in PBS buffer comparable to the most resistant *L. monocytogenes* strains (**Chapter 1**). Therefore, the strain was included in the subsequent experiments in meat matrices. Again, *L. innocua* UBU was grouped in the most pressure-resistant cluster in cooked ham as well as cured ham (**Chapter 2)**. Based on these findings, in **Chapter 3**, we compared the inactivation values of *L. innocua* UBU to the cocktail of five pressure-resistant *L. monocytogenes* strains. The *L. innocua* strains presented similar to and lower values of inactivation than the *L. monocytogenes* cocktail in the nine meat matrices evaluated and at all holding times at 600 MPa. According to our results *Listeria innocua* UBU is a suitable surrogate microorganism for *L. monocytogenes* in a wide range of high pressure-processed meat products.

The study about strain variability in the pressure-resistance of *L. monocytogenes* permitted us to suggest a cocktail composed by five pressure resistant strains, which can be used in challenge testing of high pressure-processed RTE cooked and cured meat products, and the validation of *L. innocua* UBU as a surrogate for using in in-plant validation tests or preliminary studies before challenge testing with pathogenic strains. Two approaches, challenge tests and in-plant validation, can be used for the design of safer RTE meat products through high pressure processing technology.

Conclusions and Future perspectives



Conclusions

General conclusion

The response of *Listeria monocytogenes* to high pressure processing differs strain-to-strain. This variability is influenced by the characteristics of the food matrix and the processing conditions, pressure and holding time.

Specific conclusions

1. The response, inactivation or sub-lethal injury, of *Listeria monocytogenes* to high hydrostatic pressure in a buffer system differs strain-to-strain. These differences in pressure-resistance are prominent at intermediate processing conditions. There is no correlation between the pressure-resistance of the strains and their geno- and phenotypical characteristics (lineage, serogroup, virulence-associated factors, origin and listeriosis-case history).

2. The lethal effect of high hydrostatic pressure on *Listeria monocytogenes* varies among strains in meat matrices. Holding time at 600 MPa as well as the meat matrix affect the strain variability. Pressure-resistance is not correlated with the geno- and phenotypical characteristics of the strains (lineage, serogroup, virulence-associated factors, origin and listeriosis-case history).

3. *Listeria innocua* UBU is suitable to be used as a surrogate of *Listeria monocytogenes* in validation studies in high pressure-processed RTE meat products. The proposed five-strain cocktail of *Listeria monocytogenes* is valid to be used in challenge test studies in meat products except dry-fermented meats.

Conclusiones

Conclusión general

La respuesta de *Listeria monocytogenes* al tratamiento por altas presiones difiere cepa a cepa. Dicha variabilidad depende de la matriz alimentaria y de las condiciones de procesamiento, presión y tiempo.

Conclusiones específicas

1. La respuesta, inactivación y daño sub-letal, de *Listeria monocytogenes* a las altas presiones difiere cepa a cepa en buffer. Estas diferencias en la resistencia a la alta presión son más notables en las condiciones de procesamiento intermedias. No existe correlación entre la resistencia a la presión de las cepas y sus características geno- y fenotípicas (linaje, serogrupo, factores de virulencia, origen y casos de listeriosis asociados).

2. El efecto letal de la alta presión sobre *Listeria monocytogenes* varía entre cepas en matrices cárnicas. Tanto el tiempo de procesamiento como el tipo de matriz cárnica afectan a dicha variabilidad. No existe correlación entre la resistencia a la presión de las cepas y sus características geno- y fenotípicas (linaje, serogrupo, factores de virulencia, origen y casos de listeriosis asociados).

3. *Listeria innocua* UBU se muestra adecuada para ser empleada como *surrogate* de *Listeria monocytogenes* en estudios de validación de productos cárnicos procesados por altas presiones. El cóctel de *Listeria monocytogenes* propuesto en este trabajo es válido para ser usado en *challenge tests* de productos cárnicos con la excepción de cárnicos curados-fermentados.

Future perspectives

This PhD thesis brought new knowledge about the control of *Listeria monocytogenes* by high pressure processing, resulting in the three conclusions previously stated. In addition, this new knowledge has brought new questions and curiosities, which will be the starting point of future projects:

1. Could the strain variability of *Listeria monocytogenes* to high hydrostatic pressure be explained from a genetic and transcriptomic standpoint? Do "pressure-resistant markers" exist in *Listeria monocytogenes* and other microorganisms?

2. Could *Listeria innocua* UBU be used as surrogate of *Listeria monocytogenes* strains in high pressure-processed food matrices other than meat products? Are there other pressure-resistant *Listeria innocua* strains suitable to be used as surrogates?

3. Would it be possible to design a cocktail of *Listeria monocytogenes* suitable to be used in challenge tests of different types of high pressure-processed meat products, including dry-fermented meats?

Perspectivas de futuro

Junto con las tres conclusiones enumeradas anteriormente, la presente Tesis Doctoral ha desencadenado una serie de interrogantes que serán la base de futuros proyectos:

1. ¿Podría explicarse la variabilidad en la respuesta de *Listeria monocytogenes* al tratamiento por altas presiones desde un enfoque genético y transcriptómico? ¿Existen "marcadores de resistencia a la presión" en *Listeria monocytogenes* y otros microorganismos?

2. ¿Podría utilizarse *Listeria innocua* UBU como *surrogate* de *Listeria monocytogenes* en matrices alimentarias procesadas por altas presiones distintas a productos cárnicos? ¿Existen otras cepas de *Listeria innocua* resistentes a la presión y adecuadas para ser empleadas como *surrogates*?

3. ¿Sería posible diseñar un cóctel de *Listeria monocytogenes* adecuado para ser empleado en *challenge tests* de distintos tipos de productos cárnicos procesados por altas presiones, incluidos los cárnicos curados-fermentados?

Agradecimientos / Acknowledgements

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"So — what is the meaning of life? I think people ask that question on the assumption that 'meaning' is something you can look for and go, 'Here it is, I found it. Here's the meaning. I've been looking for.' That scenario, however, doesn't consider the possibility that 'meaning' is something you create. You manufacture it for yourself and for others.

So when I think of 'meaning' in life, I ask, 'Did I learn something today that I didn't know yesterday, bringing me a little closer to knowing all that can be known in the universe?' If I live a day and I don't know a little more than I did the day before, I think I wasted that day. So the people who, at the end of the school year, say 'The summer! I don't have to think anymore!' — I just don't know. To think brings you closer to nature. To learn how things work gives you power to influence events. Gives you power to help people who may need it — to help yourself and your trajectory.

So when I think of the meaning of life, that's not an eternal and unanswerable question — to me, that's in arm's reach of me every day. So to you, at age six-and-three-quarters, may I suggest that you explore nature as much as you possibly can. And occasionally that means getting your clothes dirty because you might want to jump into puddles and your parents don't want you to do that. You tell them that I gave you permission."

- Neil DeGrasse Tyson to a 6 ¾ years old kid

El doctorado es el grado más alto que se puede conferir a nivel académico, y durante dicha formación, he tenido la oportunidad de ser alumno de diversos profesores, unos de bata blanca y otros sin ella, quienes han aportado en mi formación, personal y profesional. De uno de ellos, tan sabio como castellano, aprendí que "es de bien nacido ser agradecido", así que mi agradecimiento va dirigido a todos mis profesores, quienes me han enseñado tanto en estos años.

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Durante este tiempo he tenido que adquirir nuevas habilidades, pero también aplicar todo el conocimiento adquirido durante mis años de colegio y universidad. Por lo que quiero agradecer a todos mis profesores del Liceo de Cervantes, a los profesores de la Universidad Nacional por darme las herramientas necesarias para afrontar cualquier problema. A Carlitos y a David, porque cuando he tenido que aplicar el conocimiento ingenieril vienen a mi mente los momentos vividos y lecciones aprendidas durante la carrera ¡Gracias amigos por estar ahí!

Celia, no, no me he equivocado de lado del Atlántico, pero al ser mi profe favorita te corresponde ir aquí. ¡Ayyyyyyy, cuántas cosas he aprendido de ti, casi tantas como cenas te debo! Gracias por hacer que cada día aprenda algo nuevo, por compartir tu sabiduría proverbial castellana, el modo correcto de lavar cacharros de laboratorio, y que "n" puede ser mayor o igual que 6. Carrillo, de ti he aprendido que un "no sé" es una respuesta válida pero con fecha de caducidad, que de una aceituna a un cerdito sólo hay unos pocos trazos, que hay que ser justo y hacer siempre lo correcto (aunque muchas veces cueste), y que hay "gente con buen corazón". Gracias por el día a día y todas las lecciones que me das, las mismas en las que radica el "sentido" de la vida. Gracias a mi profe favorita por enseñarme qué significa e implica la palabra AMISTAD, por hacer de mí un mejor científico y una mejor persona. Espero seguir "aburriéndome" junto a ti durante mucho tiempo.

Cecilia Payne dijo: "La recompensa del científico joven es la emoción de ser la primera persona en la historia del mundo que ve o entiende algo. Nada se puede comparar con esa experiencia."

Gracias a todos ustedes / vosotros por ayudarme a vivir esa experiencia.

Diego

Since 1980s, after the first food-related outbreaks, the control of *Listeria monocytogenes* has become a priority for food safety authorities worldwide because of its consequences on human health.

High hydrostatic pressure, also known as high pressure processing (HPP), has demonstrated to be an efficient technology in the control of *Listeria monocytogenes*. However, its efficacy as a preservation method depends on the intra-species diversity of the target pathogen and the food matrix.

The results of this work suggested that the response of *Listeria monocytogenes* to high pressure processing differs strain-to-strain. This variability is influenced by the characteristics of the food matrix and the processing conditions, pressure and holding time.

The knowledge in strain variability allows the design of appropriate cocktails and the selection of suitable surrogate microorganisms to use in validation studies with the ultimate objective of manufacturing safer high pressure-processed meat products.

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