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Inactivation models of *Listeria monocytogenes* in raw-cured sausages submitted to high hydrostatic pressure processing and their application in a Quantitative Microbial Risk Assessment

Modelos de inactivación de *Listeria monocytogenes* en embutidos crudo-curados tratados con altas presiones hidrostáticas y su aplicación en la Evaluación Cuantitativa del Riesgo Microbiológico

Tesis doctoral presentada por

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TITULO: *Inactivation models of Listeria monocytogenes in raw-cured sausages submitted to high hydrostatic pressure processing and their application in a Quantitative Microbial Risk Assessment*

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TÍTULO DE LA TESIS: Modelos de inactivación de *Listeria monocytogenes* en embutidos crudo-curados tratados con altas presiones hidrostáticas y su aplicación en la Evaluación Cuantitativa del Riesgo Microbiológico

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INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

La presente tesis se ha desarrollado dentro del seno del Grupo de Investigación PAIDI AGR 170 (HIBRO) por los cauces normales según los procedimientos para una tesis doctoral establecidos por la Universidad de Córdoba.

La tesis encuadrada dentro del área de Microbiología predictiva, profundiza en el estudio de una nueva tecnología de conservación desarrollada en las últimas décadas, y de gran uso en el sector cárnico nacional, designada comúnmente como Altas Presiones Hidrostáticas, para su aplicación en la reducción y mitigación de riesgos microbiológicos, en especial *Listeria monocytogenes*, en productos crudos curados, donde el principal ejemplo nacional, aquí tratado, es el chorizo. Por ello, la tesis desde su planteamiento inicial representa un avance relevante de gran repercusión en el sector tecnológico, sustentada por el uso de nuevas herramientas computacionales que garantizan un menor gasto de recursos, aumenta la fiabilidad de los resultados y que la proyectan hacía su dimensión más práctica sin comprometer su rigurosidad científica.

Estructurada en 4 objetivos, la tesis se ha abordado sistemáticamente, aplicando el orden lógico establecido. En primer lugar se realizó una revisión bibliográfica exhaustiva de aquellos aspectos relevantes dentro de la aplicación de las altas presiones hidrostáticas en alimentos y contaminación cruzada, dando así cumplimento al objetivo 1 propuesto para la presente tesis. Las revisiones, publicadas en revistas de alto impacto (*Current Opinion in Food Science y*

Trends in Food Science & Technology), demuestran, por un lado, la gran capacidad de análisis y síntesis de la doctoranda, y por otro lado, la importancia de establecer una base bibliográfica en la configuración de los estudios experimentales de una tesis, como la aquí presentada. Es por tanto, de gran valor, y así debe ser reconocido, la aportación realizada por la doctoranda en este aspecto dentro de la tesis doctoral a través de su inclusión en los Capítulos I, II y V.

Desde el comienzo del doctorado el plan formativo de la tesis doctoral han llevado a la doctoranda a recibir cursos en el área de la microbiología predictiva, altamente especializados, como es el caso de *Meta-Analytical Methods in Food Safety Research*, impartido por el Instituto Politécnico de Bragança (Portugal), o la reunión formativa sobre la armonización de la Microbiología Predictiva y uso de Food-Risk lab, desarrollado en el *Federal Institute for Risk Assessment* (Alemania). Son estos y otras actividades formativas, junto a la impronta científica que caracteriza a la doctoranda lo que la ha catapultado a una dimensión extraordinaria dentro del campo de la Microbiología Predictiva.

En colaboración con el Instituto Tecnológico Agrario de Castilla y León (ITACYL), a través de la Estación Tecnológica de la Carne de Guijuelo, en colaboración con el Centro Tecnológico Empresarial Agroalimentario CTIC-CITA y dentro del proyecto de investigación Evaluación Cuantitativa del Riesgo de contaminación, supervivencia e inactivación de *Listeria monocytogenes* en embutidos crudo curados RTA 2013-00055-C02, financiado por el Ministerio de Economía y Competitividad de España, se dio la oportunidad a la doctoranda de obtener datos experimentales sobre el efecto de la tecnología de altas presiones hidrostáticas sobre la inactivación y viabilidad del patógeno *Listeria monocytogenes*. Los datos recogidos por la doctoranda a través de diferentes estancias cortas, financiadas por el citado proyecto, fueron base para el desarrollo de dos modelos de microbiología predictiva, que se han publicado en sendas revistas científicas de alto impacto, esto es, *Innovative Food Science & Emerging Technologies* y *Food Microbiology*. Estas contribuciones, además de presentar de manera brillante la confección y validación de los modelos de inactivación para su aplicación en altas presiones y productos crudos curados, representan en su conjunto un enfoque más que interesante sobre cómo se debe abordar un proceso de generación de modelos desde una

perspectiva de optimización, sentando las bases para futuros estudios en este campo. Estas aportaciones vienen recogidas en los Capítulos III y IV de la presente tesis.

La capacidad de la doctoranda para establecer colaboraciones en el ámbito internacional ha quedado más que patente a través de su participación en congresos internacionales desde el comienzo de su periplo doctoral, siendo destacables sus comunicaciones orales en ICFT (Turquía, 2018), EFSA-RARA (Holanda, 2018), Q-Safe (Grecia, 2017) y IAFP Europe (Hungría, 2014). Como culmen de ello, y gracias a la acción COST CA15118, donde el grupo de investigación AGR 170 participa activamente, la doctoranda realizó una estancia científica en la Universidad de Malta (Malta), con la supervisión de un experto en el área de la Microbiología Predictiva, y con dilatada experiencia en las altas presiones hidrostáticas, el Prof. Dr. Vasilis Valdramidis. Esta estancia, por su duración (Enero-Abril 2018) ha permitido que la presente tesis pueda llevar la *Mención Internacional*. Además, la estancia sirvió para reforzar el objetivo 3 de la presente tesis, abordando un estudio de Evaluación Cuantitativa de Riesgos Microbiológicos, enfocado a estudiar el impacto de las altas presiones, sobre el riesgo de listeriosis en productos crudos curados. Es reseñable la gran capacidad de la doctoranda, demostrada en este trabajo, para combinar conocimientos de microbiología de alimentos, tecnología alimentaria, matemáticas aplicadas, y metodologías de Evaluación del Riesgo. Aspectos que implican disciplinas diferentes, y que hacen única a la doctoranda en cuanto a su capacitación para desenvolverse en un área, que a pesar de ser incipiente, presenta una gran complejidad. El trabajo ha sido enviado para su publicación en una revista tecnológica de gran impacto en el área, *Innovative Food Science & Emerging Technologies*, estando actualmente en proceso de revisión.

El objetivo 4 fue, a su vez, acometido mediante el estudio de Evaluación de Riesgos, descrito previamente, donde la aplicación de técnicas de sensibilidad permitió evaluar la mejor configuración de parámetros de procesos de las tecnologías y formulaciones para el control de *L. monocytogenes* en chorizo, objeto de la presente tesis. Objetivo 3 y 4 se abordan en la presente tesis a través de los Capítulos V y VI.

Tanto los modelos generados en el objetivo 2, como el modelo de riesgo derivado del objetivo 3 y 4, son por si solos elementos transferibles al sector industrial nacional e internacional. La utilización de la aplicación MicroHibro (www.microhibro.com), un software desarrollado por el grupo de investigación y la propia doctoranda, es la plataforma que garantiza y canaliza la aplicación de estos resultados al tejido productivo, proporcionando una herramienta efectiva y fiable para la gestión del riesgo alimentario.

La doctoranda, con una dedicación plena y entusiasta en el desarrollo de la presente tesis, ha demostrado una gran capacidad de trabajo y de aprendizaje que le ha permitido desarrollar unas habilidades y adquirir unos conocimientos de gran valor dentro del campo científico donde ha llevado a cabo su trabajo. Son estos logros, extraordinarios, una garantía de éxito para su futuro profesional, perfilándola como una futura investigadora prominente del área de la Microbiología Predictiva, como ya queda patente en cada dato, redacción, modelo y revisión que conforman la presente tesis. Es por todo ello, que no hay otra manera de finalizar el presente informe que agradeciendo a la doctoranda haber confiado en el grupo de investigación HIBRO para desarrollar su tesis doctoral, y sintiéndonos tremendamente orgullos de su consecución brillante, que sin duda traerá, en breve, sus frutos científicos y profesionales.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 30 de noviembre de 2018

Firma del/de los director/es

Fdo.: Rosa María García Gimeno

Fdo.: Fernando Pérez Rodríguez

Agradecimientos

Gracias a todas aquellas personas que han hecho posible la realización de esta tesis y que me han dedicado lo más precioso que uno puede poseer: su *tiempo*.

Mis más sinceros agradecimientos a mi familia por creerme y apoyarme, aunque eso signifique renunciar a mi compañía.

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Al profesor Vasilis Valdramidis por permitirme realizar mi estancia predoctoral junto a él en la Universidad de Malta.

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Al Ministerio de Economía y Competitividad de España (RTA 2013-00055-C02), a la Universidad de Córdoba y a la COST Association por concederme el soporte financiero necesario para la realización de esta tesis.

Muito obrigada!

To the women I love the most

Maria de Lourdes and Ana

Às mulheres que eu mais amo

Com carinho lhes dedico esta tese

SUMMARY

Listeria monocytogenes is a foodborne pathogen of special concern in raw-cured sausages due to its capacity to survive at low pH and water activity. The ability of the pathogen to adhere and form biofilms in food-contact surfaces is also a matter of concern since cross-contamination is likely to occur in Ready-to-Eat products that are not submitted to a lethality treatment prior to consumption. In this thesis, the high hydrostatic pressure (HHP) processing technology is studied, by means of Predictive Microbiology models, as an effective non-thermal pasteurization technology to assure the compliance of the current European microbiological criteria concerning *L. monocytogenes* in raw-cured sausages. To this end, in the first instance, two systematic reviews were carried out, shedding light upon the most important factors governing cross-contamination phenomena and HHP lethality, and whose results are presented in Chapters I and II of this thesis. Based on the reviews and generated experimental data, predictive microbiology models describing the inactivation of *L. monocytogenes* by HHP in food model systems of raw-cured sausages and *chorizo* were developed as a function of the technological parameters and intrinsic factors (Chapters III and IV). These models were combined with existing scientific data and predictive models and integrated into a microbial risk assessment framework, to quantitatively assess the real impacts of nitrite reduction, cross-contamination and HHP technology application on *L. monocytogenes* levels in raw-cured sausages throughout the production and distribution chain (Chapters V and VI). Results of this thesis provides with novel and relevant information that can be used as basis to assist the application of HHP technology at the industrial level and to provide food business operators with suitable quantitative tools to ensure the microbiological safety of raw-cured sausages.

Keywords: non-thermal technologies, cross-contamination, monte-carlo analysis, experimental design, nitrite reduction, ready-to-eat foods, predictive microbiology, microbial risk assessment.

RESUMEN

Listeria monocytogenes es un patógeno alimentario de especial relevancia para los embutidos crudo-curados debido a su resistencia a condiciones de pH y actividad de agua baja. Asimismo, la alta capacidad del patógeno para adherirse y formar *biofilms* en las superficies de contacto con alimentos lo sitúa como un patógeno susceptible de contaminación cruzada en productos listos para el consumo, como es el caso de los embutidos crudo-curados. En esta tesis se ha abordado, mediante modelos de Microbiología Predictiva, el estudio de la tecnología de altas presiones hidrostáticas (APH), como un tratamiento postproceso eficaz para garantizar el cumplimiento, en embutidos crudo-curados, del criterio microbiológico sobre *L. monocytogenes* establecido por la legislación Europea. Con el fin de establecer una base de conocimiento previa se ha llevado a cabo sendas revisiones sobre modelos de APH y contaminación cruzada incluidas en los Capítulo I y II de esta tesis. Además, basados en las revisiones y datos experimentales generados en la tesis se desarrollaron modelos predictivos de inactivación de *L. monocytogenes* por APH en sistemas modelo de embutidos crudo-curados y en chorizo, considerando como variables los parámetros tecnológicos y los factores intrínsecos de dichos productos (Capítulos III y IV). Estos modelos, junto a datos y modelos predictivos extraídos de la literatura científica se integraron en un esquema de Evaluación del Riesgo Microbiológico para estimar el riesgo de exposición a *L. monocytogenes* en embutidos crudo-curados a lo largo de la cadena de producción y distribución, considerando el impacto de la reducción de nitritos, la contaminación cruzada y la aplicación de la tecnología de APH (Capítulos V y VI). Los resultados de este estudio proporcionan una fuente de información relevante y novedosa que puede utilizarse como base para la optimización de la aplicación de la tecnología de APH a nivel industrial proporcionando a los operadores de empresas alimentarias herramientas cuantitativas útiles para garantizar la seguridad microbiológica de los embutidos crudo-curados**.**

Palabras-clave: tecnologías no térmicas, contaminación cruzada, análisis de Monte-Carlo, diseño experimental, reducción de nitrito, alimentos listos para el consume, microbiología predictiva, evaluación de riesgos.

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LIST OF ABBREVIATIONS

ADI	Acceptable Daily Intake
AESAN	Spanish Food Safety Authority
ANICE	National Meat Processing Industries Association Of Spain
ANOVA	Analysis Of Variance
ASZ	Acceptable Simulation Zone
BHIA	Brain Heart Infusion Agar
BHIB	Brain Heart Infusion Broth
CAC	Codex Alimentarius Commission
CCD	Central Composite Design
CECT	Spanish Type Culture Collection
CFU	Colony Forming Unit
CRA	Chemical Risk Assessments
EC	European Commission
EFSA	European Food Safety Authority
EU	European Union
FAO	Food And Agriculture Organization Of The United Nations
FDA	Food And Drug Administration
FSIS	Food Safety And Inspection Service (United States Department Of Agriculture)
FSO	Food Safety Objective
HACCP	Hazard Analysis And Critical Control Points
HHP	High Hydrostatic Pressure
IPCS	International Program Of Chemical Safety
IRTA	Institute Of Agrifood Research And Technology
ISO	International Standard Organization
LSD	Least Significant Difference
MEA	Microbial Exposure Assessment
MRA	Microbial Risk Assessment
PCA	Plate Count Agar

PD	Purchase Day
PM	Predictive Microbiology
PRE	Proportion Of Relative Errors
QMEA	Quantitative Microbial Exposure Assessment
QMRA	Quantitative Microbial Risk Assessment
RAKIP	Risk Assessment Modelling And Knowledge Integration Platform
RBA	Risk-Benefit Assessment
RH	Relative Humidity
RMSE	Root Mean Square Error
ROS	Reactive Oxygen Species
RSM	Response Surface Methodology
RTE	Ready-To-Eat
SD	Standard Deviation
SMM	Simulated Meat Medium
TDI	Tolerable Daily Intake
TR	Transfer Rate
TSB	Tryptone Soy Broth
TTI	Time To Inactivate
TTP	Total Transfer Potential
UBD	Use-By-Date
WGS	Whole Genome Sequencing
WHO	World Health Organization

1. Introduction

Raw-cured sausages are traditional dry-fermented products manufactured using traditional technologies and widely consumed in Spain (Toldrá, 2015). They are generally formulated with pork meat, fat, spices and salt. Traditionally, curing agents such as nitrites and/or nitrates are added to formulations to inhibit growth of undesirable bacteria and to help on the development of the typical organoleptic properties of these products (Sidira, Kandylis, Kanellaki, & Kourkoutas, 2015). However, due to the increase in health-conscious, low salt foods or foods exempt of chemical additives, such as nitrite, have gradually gained attention among consumers, which demand products with better quality, improved food safety, nutritional value and freshness (Huang, Wu, Lu, Shyu, & Wang, 2017).

Listeria monocytogenes, a pathogen of concern in the meat industry due to its ubiquitous and psychrotrophic nature, has been frequently isolated from raw-cured sausages and their processing environments (Gounadaki, Skandamis, Drosinos, & Nychas, 2008; Martin, Garriga, & Aymerich, 2011). Even if pathogen concentration in these products is low, post-processing cross-contamination phenomena can increase its prevalence and levels to values considered sufficient to cause human listeriosis (de Candia, Morea, & Baruzzi, 2015). For instance, an outbreak of human listeriosis linked to the consumption of luncheon meats contaminated during the slicing operation at the meat industry resulted in 22 deaths in Canada, in 2009 (Anonymous, 2009).

Since *L. monocytogenes* is strongly influenced by the presence of nitrites in foods, the absence/reduction of this chemical additive could affect the microbiological stability of raw-cured sausages (Christieans, Picgirard, Para, Lebert, & Gregori, 2018; Hospital, Hierro, & Fernández, 2012). In this context, the development of emerging non-thermal processing technologies, enabling the reduction of additives in foods, without affecting negatively their quality and safety has been encouraged (Holck, Axelsson, Mcleod, Rode, & Heir, 2017).

Non-thermal technologies, such as pulsed electric field, pulsed light, electron beam and plasma have been intensively investigated as promising alternatives to the traditional heat processing, which affects negatively the sensory characteristics, flavours, vitamins and consequently, the nutritional content of foods (Barba, Koubaa, Do Prado-Silva, Orlien, & Sant'Ana, 2017). However, high hydrostatic pressure (HHP) is the most successfully commercialized emerging non-thermal technology (Huang et al., 2017). During the application of HHP, foods are submitted to high levels of pressures for some minutes, which cause structural damages in microbial cells, and consequently, their inactivation (Ferreira, Almeida, Delgadillo, Saraiva, & Cunha, 2016; Syed, Buffa, Guamis, & Saldo, 2016).

The European Commission (EC) Regulation 2073/2005 requires food manufacturers to demonstrate that *L. monocytogenes* levels in RTE foods will not exceed 100 cfu/g throughout their shelf-life. The microbiological criteria are stricter in some countries such as the United States and Japan, which requires absence of the pathogen in 25 g of product (FSIS, 2003). Predictive microbiology tools are proposed as suitable tools to support the optimization of HHP application, assisting the compliance of microbiological criteria. In general, mathematical models can be used to identify factors influencing the microbial behaviour associated with any food process, including novel processing technologies, and understand attendant microbial dynamics (Bover-Cid, Belletti, Garriga, & Aymerich, 2011; Pérez-Rodríguez, Valero, Carrasco, García-Gimeno, & Zurera, 2008). When integrated into Microbial Risk Assessments (MRA), predictive microbiology models can help us to determine the impacts of reformulation, cross-contamination and the application of novel technologies on *L. monocytogenes* concentration and prevalence in raw-cured sausages as well as in the final risk associated to their consumption.

In **Chapter I** of this PhD thesis an updated overview of available cross-contamination modelling approaches in foods as well as the available evaluation methods of model robustness are provided. Furthermore, the factors implicated in the modelling of cross-contamination dynamics in food processing environments are illustrated, describing the underlying phenomena of transfer and survival of pathogens in foods.

To support the practical application of pressurization of foods as a non-thermal pasteurization method, an exhaustive review of predictive microbiology models of *L. monocytogenes* behaviour in foods submitted to HHP technology is presented in **Chapter II**. A description of the main factors influencing on the lethality of this technology is also presented, including technological parameters, food matrix characteristics and *L. monocytogenes* culture conditions.

In **Chapter III** a mathematical approach of *L. monocytogenes* inactivation in a simulated meat medium (SMM) during the application of HHP technology is addressed. Since the development of product-oriented modelling approaches can be very-time consuming and expensive, the SMM developed mimic a wide variety of meat products, including raw-cured sausages. The influence of pH, NaCl content and $NaNO_2$, as well as the influence of the technological parameters pressure and pressure-holding time on process lethality was evaluated.

A product oriented-approach is presented in **Chapter IV,** with the development of an inactivation model of *L. monocytogenes* based on data obtained during pressurization of Spanish chorizo sausage without added nitrates and nitrites. In accordance with literature data, the relevant factors taken into account for model development were: pressure, pressure-holding time and the water activity (a_w) of the sausages. In this approach, the impact of sausages reformulation regarding the presence of chemical additives in parallel with the application of HHP technology on food safety concerning *L. monocytogenes* was evaluated.

Besides innovative predictive microbiology models, this PhD thesis presents an example of their application in the context of a MRA, through the performance of a Quantitative Microbial Exposure Assessment (QMEA). The concepts and foundations of MRA are introduced in **Chapter V**. Finally, in **Chapter VI** a probabilistic model, based on previous predictive microbiology models, is designed and simulated to predict changes in *L. monocytogenes* concentration during the production and distribution chain of Spanish chorizo sausages, subjected to HHP processing, taking into account sausages reformation (*i.e.*, nitrite reduction) and the possibility of cross-contamination occurrence during post-processing operations and sausages.

2. Fundamental concepts

In this section, key concepts and fundaments of relevance to the present PhD thesis are briefly presented.

2.1. *Raw-cured sausages*

Raw-cured sausages, also known as dry-fermented sausages, are uncooked meat products composed by mixtures of minced lean meats and fatty tissues combined with salts, spices, nitrites and sugar, subsequently filled into casings. Their characteristic organoleptic properties are developed through a fermentation process, that can be assisted or not by starter cultures, followed by a drying process to reduce their water content (Toldrá, 2015). These products are not submitted to any heat treatment during processing and are in most cases distributed and consumed raw (FAO, 2010). Besides, these products are usually encountered sliced and vacuum-packed, due to consumer convenience, marketing and quality reasons. Nowadays, a large number of different raw-cured sausages are produced widely using different recipes (Toldrá, 2015). *Salchichón*, *chorizo*, *fuet* and salami are included in this category of meat products (Yilmaz & Velioglu, 2009).

Fermented raw-cured sausages are highly treasured foods in Spain, with elements of culinary heritage and identity. The Spanish production of raw-cured sausages in the last five years is represented in Figure 1. They were traditionally considered healthy products, as meat is especially rich in proteins, vitamins and minerals. Besides, these products were considered safe from the microbiological point of view, due to their physicochemical characteristics (*i.e.*, low pH attributed to fermentation, 4.6-5.3, and $a_w \leq 0.92$ due to salting and drying). More recently, consuming fermented sausages has been associated with health issues caused by the high contents of saturated fats and Na (Holck et al., 2017). The presence of nitrite as a curing agent in fermented sausages is also a matter of concern, as it can originate nitrosamines, compounds with recognized carcinogenic activity (De Mey et al., 2014; Hospital et al., 2012).

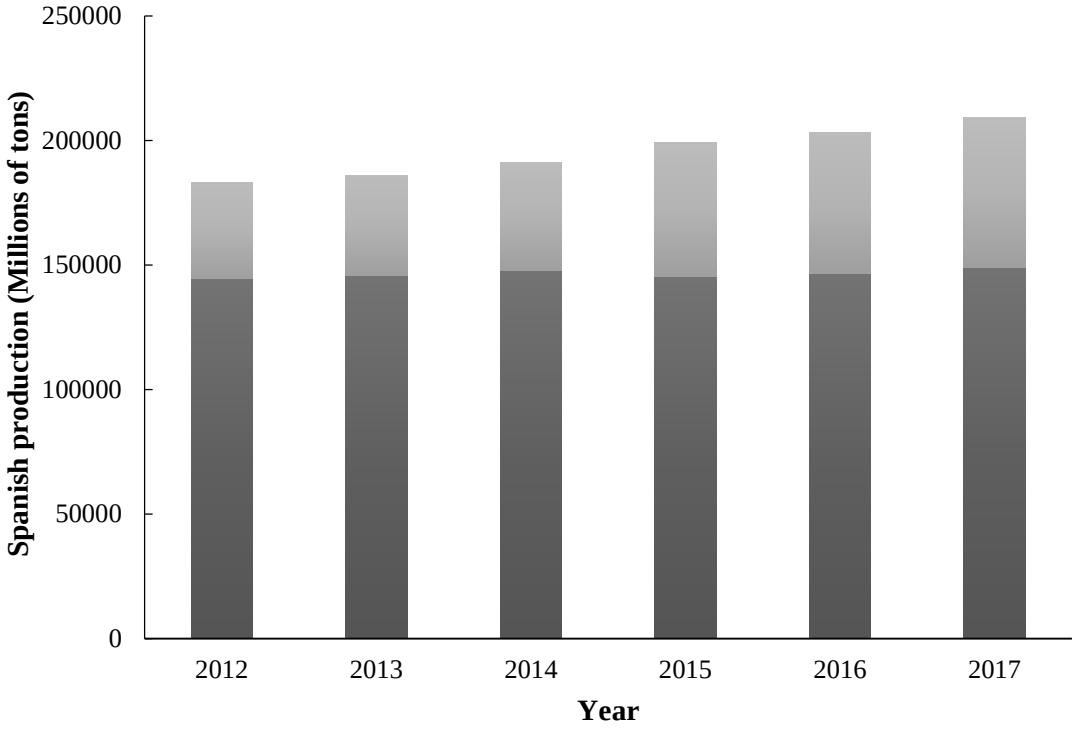

Figure 1- Raw-cured sausages production in Spain: (■) National consumption and (■) Export *(Source: ANICE, 2018)*

The occurrence of several outbreaks linked to the consumption of dry or semidry fermented sausages have demonstrated that their manufacturing processes do not ensure microbiologically safe products (Holck et al., 2011; Kuhn, Torpdahl, Frank, Sigsgaard, & Ethelberg, 2011; Pierre, 2015). Surveys have reported the presence of pathogenic *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* Typhimurium and *Listeria monocytogenes* in fermented sausages, besides reporting *Clostridium botulinum* and *Toxoplasma gondii* as potential microbial hazards associated with the consumption of these products (Holck et al., 2017).

2.2. *Listeria monocytogenes*

Listeria monocytogenes is a psychrotrophic, gram-positive, pathogenic microorganism, which has emerged as one of the main foodborne pathogens, causing several fatal outbreaks of illness worldwide (Buchanan, Gorris, Hayman, Jackson, & Whiting, 2017). It is widely distributed in

the environment and can grow/survive in a wide range of conditions, such as high salt or low pH, as well as in low humidity and oxygen environments (Buchanan et al., 2017). Besides being present in the air, soil, water, dust and plant material, *L. monocytogenes* is a transitory resident of the intestinal tract in humans and 2-10% of the general population carries the microorganism without any apparent health consequences (Buchanan et al., 2017; FSIS, 2014).

Foodborne listeriosis is primarily acquired through consumption of contaminated foods, particularly those ready-to-eat (RTE), including processed foods that have been exposed to the processing environment after application of a lethality treatment prior to packaging (Buchanan et al., 2017; Fang, Liu, & Huang, 2013). Meat products with relatively long shelf-life, including fermented salami, are mainly included among the foods implicated in listeriosis cases worldwide (Nastasijevic et al., 2017).

The vast majority of listeriosis cases occurs in young, elderly, pregnant women or immunocompromised individuals, leading to spontaneous abortion, septicemia, meningitis or other infections of the central nervous system, and even death (Ferreira, Wiedmann, Teixeira, & Stasiewicz, 2014). In healthy individuals, listeriosis usually causes flu-like symptoms (Ferreira et al., 2014; FSIS, 2014). According to the most recent report of the European Food Safety Authority (EFSA), there was an increasing trend of listeriosis from 2008 to 2016. Among the zoonoses, listeriosis caused the highest hospitalization and mortality rate in 2016, corresponding to 16.2% among the 2,536 confirmed cases in the European Union (EU) (EFSA, 2017).

L. monocytogenes can enter food processing environments through raw materials, food handlers and the movement of equipment (Buchanan et al., 2017). Despite of aggressive cleaning and sanitization procedures, the pathogen can persist and form biofilms in different inaccessible locations of food-contact surfaces (González-Rivas, Ripolles-Avila, Fontecha-Umaña, Ríos-Castillo, & Rodríguez-Jerez, 2018). Many authors have confirmed the ability of *L. monocytogenes* to adhere in processing facilities and to form biofilms in food-contact surfaces, such as plastic, polypropylene, rubber, stainless steel, and glass (de Candia et al., 2015; E. E. Giaouris & Simões, 2018; Kocot & Olszewska, 2017). Thus, minimizing risk by *L. monocytogenes* requires a tight control by food risk managers.

Cross-contamination in meat processing environments is considered one of the main sources of contamination by *Listeria monocytogenes* (Pérez-Rodríguez et al., 2010). The important role of contaminated processing equipment and environments as a source of listeria to RTE meats has been indicated in several studies (Giaouris et al., 2013; Gounadaki et al., 2008; Thévenot, Delignette-Muller, Christieans, & Vernozy-Rozand, 2005).

Regarding fermented sausages, Cadavez et al., (2016) found out that cross-contamination, especially during mixing of raw materials, should be avoided as it is an important factor explaining the increase in *L. monocytogenes*, *Enterobacteriaceae*, and *S. aureus* in a naturally fermented Portuguese sausage on a batch basis. Furthermore, studies have shown that slicing machines are important sources of *L. monocytogenes* (Borovic et al., 2014; Lin et al., 2006; Vorst, Todd, & Ryser, 2006) and the transfer ability of the pathogen during the slicing operation of raw-cured sausages and other deli meats have also be proven (Chaitiemwong, Hazeleger, Beumer, & Zwietering, 2014; Lin et al., 2006; Sheen & Hwang, 2008; Vorst et al., 2006).

Raw-cured sausages are considered to be products of low to moderate risk associated with listeriosis (Holck et al., 2017). However, taking into account the high variety of recipes and processing conditions (*i.e.*, fermentation and drying process) and the possibility of cross-contamination occurrence during post-processing operations, it is difficult to ensure that all products comply with the European microbial criterion for RTE foods which is ≤ 100 cfu/g (European Commission, 2005) and also with the microbial criterion of importing countries such as the United States, in which a "zero tolerance" for the presence of the pathogen is applied (FSIS, 2003). It is therefore necessary to gather information on the safety of raw-cured sausages in terms of *L. monocytogenes* growth/survival and implement processing strategies to assure food safety.

2.3. *High hydrostatic pressure processing*

High hydrostatic pressure (HHP) processing is a non-thermal food preservation technology, suitable to eliminate post-processing pathogen contamination in foods, without causing severe impacts on their organoleptic and nutritional characteristics (Syed et al., 2016). It has been

investigated as a suitable alternative to the application of heat processing and consists of the application of isostatic pressures in foods (200-800 MPa, in some cases up to 1000 MPa) for some minutes, which are transmitted by air-driven pumps through water (Hugas, Garriga, & Monfort, 2002). Since the foods are pressurized on their final packaging, re-contamination after pressurization does not occur. A scheme of the application of high-pressures in foods is shown in Figure 2.

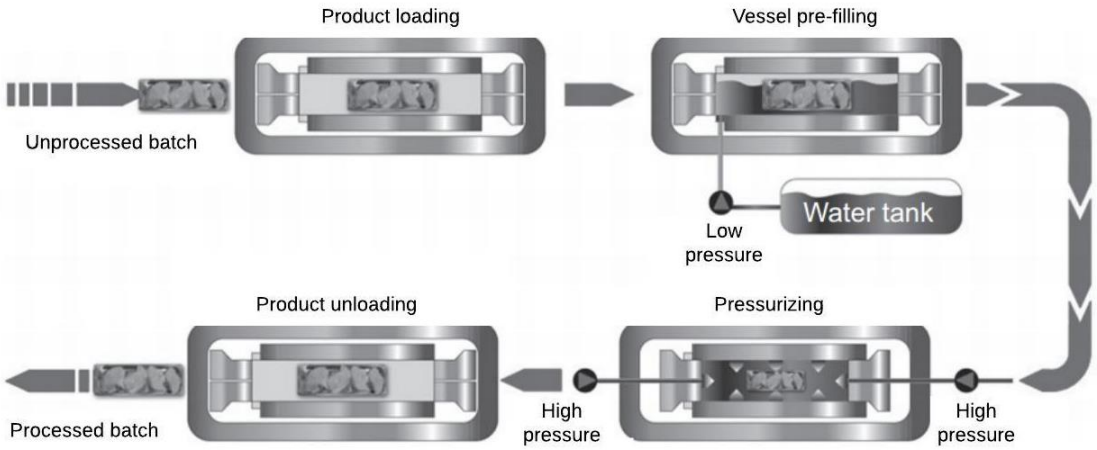

Figure 2- Scheme of high hydrostatic pressure processing (*Source: Bolumar, Georget, & Mathys, 2015)*

HHP is based on the application of two physical principles (Syed et al., 2016). The Le Chatelier`s principle explains the antimicrobial effectiveness of HHP and determines that the application of pressure enhances reactions leading to a volume decrease (*i.e.*, protein denaturation, starch gelatinization, transition of water to ice, etc.) (Campus, 2010; Speranza & Corbo, 2012). As a result, microbial cell components are affected by high pressures, including cell membranes and their proteins, enzymes and ribosomes, and consequently, the cell metabolism (Georget et al., 2015). The second principle determines that HHP processing is not affected by the volume or the shape of foods, being the pressure uniformly and instantaneously distributed around and throughout the product (*i.e.,* isostatic rule) (Speranza & Corbo, 2012; Syed et al., 2016).

The HHP technology was first applied by Bert Hite for milk preservation in the 1890s (Hite, 1899). Despite of his initial efforts, it was only in the 1990s that HHP-treated products started to be commercialized in Japan, more specifically jams and jellies (Speranza & Corbo, 2012). Currently, HHP-treated products can be found in the market of many countries, including Romania, Netherlands, Greece, United States and Spain, thanks to the existence of industrial equipment from different manufacturers (Huang et al., 2017). In Spain, high-pressured raw-cured products including ham and chorizo have been commercialized (Bajovic, Bolumar, & Heinz, 2012).

The commercial application of this innovative technology is supported by a number of universities, governmental departments, and research institutions that actively conduct studies on HHP application in foods to stablish a common technical standard for the pasteurization of foods, to evaluate its health and safety aspects and operational costs (Huang et al., 2017).

2.4. *Predictive microbiology*

Predictive microbiology can be defined as the gathering of different disciplines, including food microbiology, engineering and statistics, to provide useful predictions about the microbial behaviour in foods (Mafart, 2005). It is an applied research field aimed to assess quantitatively the microbial behaviour in foods through the development and application of mathematical models. A mathematical model provides an estimation of the microbial behaviour in a food (*i.e.*, response) based on environmental conditions or other factors such as its intrinsic characteristics, including a_w or pH (*i.e.*, input variables) (Pérez-Rodríguez & Valero, 2013).

Predictive microbial models describe kinetic processes such as microbial death and growth and physical processes such as microbial transfer. Probabilistic models provide estimations of the probability of a microbial process occurrence, including growth and recovery. Traditionally, models are classified in mechanistic or empirical models, based on the information used to construct the model. A model based on the understanding of the underlying phenomena governing the system is classified as mechanistic, while empirical models are generated based on system observations (Pérez-Rodríguez & Valero, 2013).

Polynomial models developed by using the Response Surface Methodology (RSM) are examples of empirical mathematical models. This methodology is one of the most popular process optimization technique in the field of food science, performed to estimate the relationship between a set of experimental factors, including technological variables and food characteristics, and observed outcomes (Barba, Criado, Belda-Galbis, Esteve, & Rodrigo, 2014; Bover-Cid, Belletti, Aymerich, & Garriga, 2015; Gao, Ju, & Wu-Ding, 2007). For instance, the microbial inactivation during the application of innovative technologies including HHP have been modelled by using RSM, as a function of the pressure intensities applied, the pressure-holding time, process temperature, and food characteristics, such as a_W and fat content (Bover-Cid et al., 2015).

Predictive microbiology kinetic models can also be classified as primary, secondary and tertiary models. The primary models describe microbial concentration versus time, whereas secondary models describe changes in primary models parameters as a function of environmental variables (Pérez-Rodríguez & Valero, 2013). Finally, tertiary models are the integration of primary and secondary models in user-friendly computational software tools, in which users can obtain predictions of microbial behaviour in foods in a quick and easy manner (Tenenhaus-Aziza & Ellouze, 2015).

For food industries, predictive microbiology models are of great interest for assuring food safety and quality. They can be applied to estimate the effects of formulation and storage factors on microbial levels in foods, for new products development and for processes optimization. As a safety management tool, predictive microbiology can assist on the compliance of microbiological criteria in foods, on the implementation of Hazard Analysis and Critical Control Points (HACCP) systems and on the performance of Microbial Risk Assessments (MRA) (Gougouli & Koutsoumanis, 2016). Predictions of spoilage or pathogenic microorganisms in foods by means of mathematical equations are also useful for determining shelf-life (Possas et al., 2018).

Predictive models are useful for estimating the microbial behaviour in foods within the ranges of the variables used for their development, but their validation prior to application is essential

to ensure reliable predictions. Nevertheless, caution must be taken for models interpretation due to the wide confidence intervals of the fitted equations representing for the uncertainty on estimates (Holck et al., 2017; Pérez-Rodríguez & Valero, 2013).

2.5. *Microbial Risk Assessment*

The field of MRA is one of the most relevant topics that have emerged over the decades concerning food microbial safety. MRA is a scientifically based framework consisting of four steps: (1) hazard identification, (2) hazard characterization, (3) exposure assessment, and (4) risk characterization (Codex Alimentarius Commission, 1999). It is performed in order to achieve a full understanding of the risk associated with a biological hazard that may be present in a food, taking into account the whole farm-to-fork production chain or part thereof that is considered relevant regarding food safety (Cummins, 2017).

Risk assessment is one of the three components of the Risk Analysis process, composed also by Risk Management and Risk Communication components (FAO/WHO, 2013). The Risk Analysis is the dominant process intended to ensure food safety and it has been created to face the issues derived from globalization of trade, which include the transmission of harmful bacteria with increased resistance along the food chain and between different countries. The four steps of a MRA are briefly described below, according to the definitions proposed by the Codex Alimentarius (1999).

Hazard Identification. "The identification of biological agents capable of causing adverse health effects and which may be present in a particular food or group of foods".

Hazard Characterization. "The qualitative and/or quantitative evaluation of the nature of the adverse health associated with the hazard".

Exposure Assessment. "The qualitative and/or quantitative evaluation of the likely intake of a biological agent via food, as well as exposure from other sources if relevant".

Risk Characterization. 'The process of determining the qualitative and/or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of

known or potential adverse health effects in a given population based on hazard Identification, Hazard Characterization and Exposure Assessment".

Predictive models can be integrated into the Risk Analysis process on the design of scientifically based management strategies to assure food safety. Furthermore, they can be applied in quantitative exposure assessment to describe prevalence and microbial population dynamics along the food chain towards a specific pathogen (Pérez-Rodríguez & Valero, 2013). An example of a process diagram for an exposure assessment model using specific predictive models for describing microorganism transmission along a food chain is shown in Figure 3.

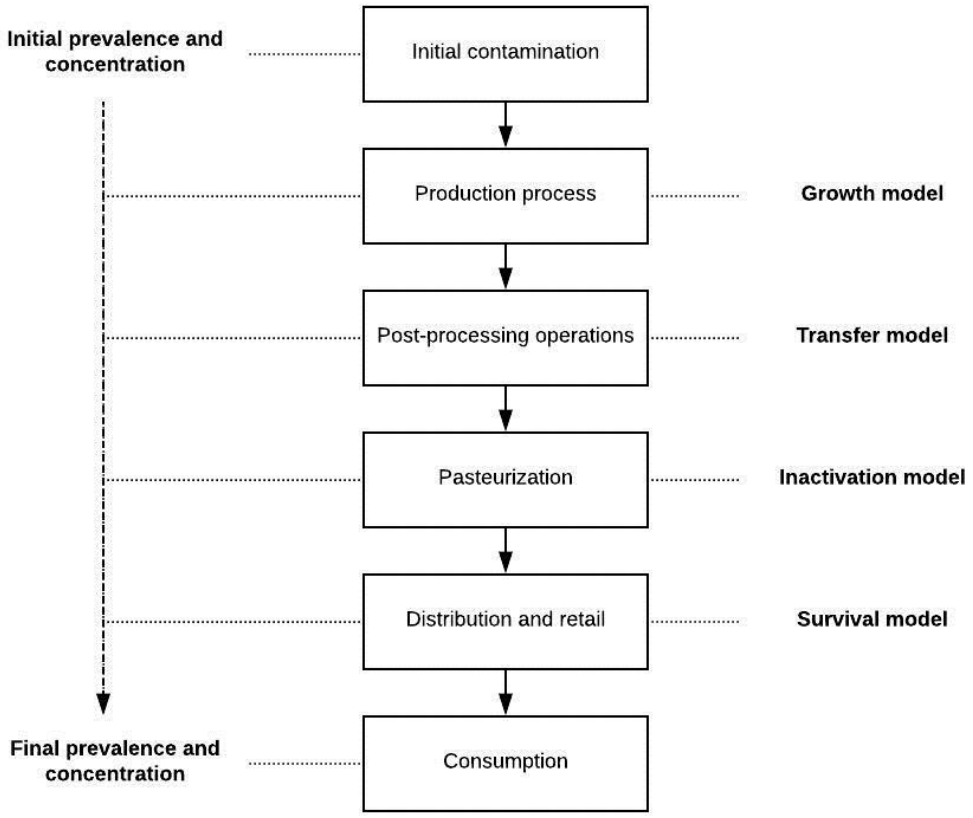

Figure 3- Process diagram of a hypothetical quantitative exposure assessment model using specific predictive models for describing microorganism transmission along the represented food chain *(Source: adapted from Pérez-Rodríguez & Valero, 2013)*

The estimation of microbial concentration and prevalence in food products by the end of the production chain or at the time of consumption is typically more relevant to the industry than the estimation of risk (*i.e.*, probability of illness or death after consuming a contaminated food). Thus, Microbial Exposure Assessment (MEA) studies are usually performed rather than a complete MRA process (Membre, 2016).

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3. Objectives

1) To provide an updated overview of available cross-contamination modelling approaches as well as existing predictive microbiology models of *L. monocytogenes* behaviour during the application of high hydrostatic pressure processing in foods *(Chapter I and II)*.

2) To develop predictive models of *L. monocytogenes* pressure-induced inactivation in raw-cured meat products with different formulations, considering the most important factors influencing on process lethality, including technological parameters and food matrix characteristics (*Chapters III and IV*).

3) To integrate the developed inactivation models into the microbial risk assessment framework, by constructing a Microbial Quantitative Exposure model to predict the fate of *L. monocytogenes* in raw-cured sausages throughout the production process and distribution chain after providing the concepts and foundations of the risk assessment in foods (*Chapter V and VI*).

4) To evaluate the impacts of changes in formulation (*i.e.*, nitrite reduction) on the safety of raw-cured sausages when HHP technology is applied as a post-lethality process to inactivate *L. monocytogenes* (*Chapter VI*).

Chapter I

Chapter I: Models of microbial cross-contamination dynamics

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1.1. Abstract

Cross-contamination can be understood as a systematic process where contaminated surfaces are involved in food contamination. However, in most cases, it is mainly referred as a sporadic event affecting the number of contaminated food samples in a lot rather than the concentration levels, since bacterial transfer often occurs at low numbers. Bacterial transfer, although recently, has been considered as an important area to be modelled and several studies have made attempts to give insight in the transfer process to provide more reliable models and predictions. The use of compartmental mechanistic models could allow to better understand the influence of food processing factors and the indirect mechanisms involved in cross-contamination.

Keywords: cross-contamination dynamics, transfer rate, compartmental mechanistic models, risk assessment, acceptable simulation zone.

1.2. Introduction

Microbial growth in foods reflects changes in concentration over time as a function of certain conditions. Over the last few years, the need to describe how microorganisms are transmitted throughout the food chain has led microbiologists to look at other bacterial processes than growth and death. Cross-contamination is reported to be an important factor strongly linked to foodborne diseases outbreaks and food spoilage. A limitation of existing predictive models in this regard is the lack of reproducibility in some cases to characterize variability associated to bacterial transfer from contaminated food surfaces to other recipient food surfaces in food-related environments. In recent years, understanding of the modelling transfer dynamics allows to provide quantifiable links between processing control parameters and microbial levels, simplifying the complexity of these relationships for implementation into risk assessment models.

The present paper aims at providing an updated overview of available cross-contamination modelling approaches in foods as well as the available evaluation methods for model robustness. Theoretical concepts are illustrated in two examples on the factors implicated in the modelling of cross-contamination dynamics in the produce and poultry production chains describing the underlying phenomena of transfer and survival of pathogens.

1.3. Overview of existing approaches of cross-contamination models in foods

Cross-contamination models have experienced a great development in the last years and different approaches have been adopted to explain the behavior of microorganisms during transfer through contact between different surfaces. Pérez-Rodríguez and others [1••] published the state-of-the-art of bacterial transfer phenomenon, including a review of the transfer models developed so far and the factors affecting cross-contamination and recontamination phenomena. In their review, it was stated that the most popular models [2, 3, 4] are based on the so-called transfer rates (TR), as can be seen in Eq. (1.1).

$$N_r = \left(\frac{TR}{100} \right) \cdot N_d \qquad \text{Eq. (1.1)}$$

where N_r is the quantity of cells transferred to the receptor surface; TR is transfer rate, i.e. the percentage of cells transferred from one surface (donor) to another surface (receptor), and N_d is the quantity of cells contaminating the donor surface.

Refinement of this simple model has been proposed by other authors, assuming that there are variability and uncertainty components inherent to bacterial cells transfer. To capture this, various probability distributions have been evaluated to model transfer. Normal distribution was considered by Montville and others [5]; Schaffner [6]; and Jensen and others [7] as the most appropriate to describe the log-transformed TR data. Other distributions (i.e. Weibull, Beta) have been suggested [8] to describe TR between different surfaces during food process operations involving handlers and semi-elaborated foods.

Other models [9•] utilize the compartmental and dynamic cross-contamination approach based on the binomial process of bacterial transfer, as described by the parameters (*n=number of samples*, *p=probability of cells transfer*). Smid and others [10] applied a Bayesian network model allowing the combination of uncertainty within one experiment and variability over multiple experiments; the posterior distribution of bacteria in the recipient surface was a Gamma distribution, while the variability of TR over all experiments was defined by a Beta distribution. The authors demonstrated the functionality of the model and provided more insight into the transfer probabilities of *Salmonella* between pork and stainless steel knife. They found a very large variability and a considerable uncertainty.

In some cases, events of 0% cells transfer between surfaces are observed. Some authors [11, 12] attempted to model failed bacterial transfers applying cross contamination frequency values and TRs to describe microbial prevalence and concentration changes, respectively. Ariza and others [9•] also explained this phenomenon by assuming very low values for the *n* and *p* parameters of the Binomial distribution.

The use of compartmental mechanistic models was illustrated by Møller and others [13] building a more complex transfer model for *Salmonella* during pork grinding inspired in a

previous model [14•] developed for *Campylobacter* cross-contamination in poultry processing. Møller and others [13] hypothesized that the input of *Salmonella* is organized in two different matrices inside the grinder; one exhibiting high transfer ability, and a second where *Salmonella* demonstrated a low transfer from the grinder to the meat. The resulting model has seven parameters with biological significance; four of them are TR and the other three are cells inactivation. Some years later, they evaluated the model developed in other grinding conditions, i.e. two microorganisms (*Salmonella* spp. and *Listeria monocytogenes*), two food matrices (pork and beef), two different grinders, different sizes and number of pieces of meats to be ground, and different temperatures [15••]. Regarding cross-contamination of pork through contact with an artificially inoculated slicing machine, other studies adjusted a log-linear model and Weibull model to transfer data, showing acceptable goodness-of-fit indexes [16] ($R^2 \geq 0.73$).

A recent study [17•] developed a mechanistic model focused on cross-contamination dynamics during produce washing, based on the previous experiments of Luo and others [18]. Munther and others [17•] provided a system of equations combining the dynamics of water chemistry and pathogen transmission from the wash water to shredded lettuce. Related also with cross-contamination via water, other complex approaches account for the transfer of *Escherichia coli* during chilling process of poultry in a water tank [19•]. It is overall concluded about the significance of processing factors on cross-contamination dynamics underlining the utility of the models proposed to quantify the effect of indirect mechanisms involved with cross-contamination [17•, 19•].

1.4. Factors involved in the modelling of contamination dynamics

Environmental and intrinsic factors during processing affect the ability of microorganisms to be transferred from one surface to another. Intrinsic factors encompass the physiological characteristics and type of microorganisms, their degree of attachment, clustering and/or biofilm forming capacities. Moistness and roughness of the donor and receiving surfaces, as well as the contact time between them can be described as relevant environmental factors [1••].

When modelling cross-contamination dynamics in foods the contamination between food-equipment, food-water, water-equipment together with the reverse scenarios should be evaluated [20, 21]. For instance, the transfer of *Salmonella* from meat to surfaces is more likely to occur when the meat skin moisture is high [22••]. The variability and uncertainty derived from the simultaneous action of several factors during transfer events make stochastic approaches using probability distributions more adequate when modeling the dynamics of cross-contamination [19•, 20].

The influence of processing factors is illustrated through the following examples:

1.4.1. Produce chain

The washing water management in fresh-cut produce lines is of great importance concerning cross-contamination [23, 24]. The binding rate, defined as the rate at which the microorganism present in the water binds to the produce, the free chlorine concentration and the washing holding time are factors that influence on the contamination dynamics [17•, 24].

Considering the cross-contamination scenarios involving transfer from equipment to food (i.e.: cutting, shredding, etc.) the physicochemical characteristics and topography of the donor and receiving surfaces were highlighted as important factors while modeling microbial transfer [25, 26•]. Zilelidou and others [21] concluded that bacterial transfer might take place with higher TR from contaminated knives to fresh lettuce compared to the reverse scenario. Furthermore, higher residence times on the donor surface leads to lower TR, as it enables internalization or attachment of bacteria [1••, 21, 27].

The physiological characteristics and the susceptibility of different microorganisms to stressful environmental conditions can considerably influence on TR [1••, 21]. *E. coli* O157:H7 was more susceptible to the desiccation stress caused by low relative humidity, with lower survival capacity on stainless steel surfaces soiled with different vegetable juice substrates in comparison with *Salmonella* spp. [28]. Lower TR for *E. coli* O157:H7 than for *L. monocytogenes* from cutting knives to lettuce were attributed to the higher susceptibility of Gram negative cells to stressful conditions [21]. Thus, differences in the bacterial survival capacity on food contact

surfaces, as affected by temperature, relative humidity or nutrient availability, confirm that the type of microorganism is an important factor when modelling cross-contamination dynamics.

The stochastic simulation of the transfer model of Pérez-Rodríguez and others [20] showed that the initial level of *E. coli* O157:H7 on a lettuce batch entering in a processing line increased significantly pathogen spreading in fresh-cut lettuce batches entering subsequently. Furthermore, regarding the influence of different initial levels of contamination on donor surfaces, Faour-Klingbeil and others [29] found out significantly higher TR from cutting boards to parsley when low contamination levels were present, which could be associated with the microbial attachment strength [1••]. These studies demonstrated the importance of considering cross-contamination to quantify microbial prevalence.

The mechanical slicing of foods also can enable bacterial transfer [30]. Wang and Ryser [31] assessed the impact of multiple process variables on the transfer of *Salmonella* Typhimurium during tomatoes slicing. Post-contamination holding time, processing temperature and slice thickness did not significantly affect the TR or the overall percentage of transferred cells. Significantly higher percentage of *Salmonella* could be transferred to tomatoes from high humidity surfaces, and the microbial levels decreased with the slice number, as shown in other studies [32].

Mean transfer rates from cross-contamination studies during fresh produce chain are represented in Table 1.1. According to the aforementioned studies, the cross-contamination phenomenon governed by a number of dynamically changing and/or unknown factors, such as changes in the hydrophobicity of cutting equipment surfaces in contact with food residues [1••] or changes in free chlorine concentration of washing water. Thus, it is difficult to identify which factors are more relevant when modelling cross-contamination dynamics and/or all the factors that simultaneously interfere on a microbial transfer.

Table 1.1- Mean transfer rates from cross-contamination studies during fresh produce chain.

Food commodity	***Microorganism***	***Processing step***	***Type of contact***	***Mean TR (%)***	***Reference***
Lettuce	*E. coli* O157	Washing	Water-Produce	1.0	[23]
	MS2 phage			0.5	
	Murine norovirus			0.5	
Lettuce	*E. coli* O157:H7	Washing	Produce-Produce	0.2	[18]
Lettuce	*E. coli* O157:H7	Shredding	Produce-Shredder	0.02	[20]
		Rinsing	Produce-Shaker	0.01	
		Centrifugation	Produce-Centrifuge	0.04	
		Washing	Produce-Water	8.79	
		-	Equipment-Produce	15.33	
Lettuce	Human norovirus	Handling	Produce-hand	1.1	[25]
			Hand-lettuce	1.1	
		Cutting	Produce-Knife	1.1	
			Knife-Produce	100.0	
			Produce-Board	2.1	
			Board-Produce	25.0	
Lettuce	*E. coli* O157:H7	Cutting	Produce-Knife	26.5	[21]
			Knife-Produce	31.5	
	L. monocytogenes		Produce-Knife	20.5	
			Knife-Produce	44.9	
Parsley	*S.* Typhimurium	Cutting	Board-Produce	1.0-64.0*	[29]
Tomatoes	*S.* Typhimurium	Mechanical Slicing	Slicer-Produce	0.0-12.2*	[31]

* Transfer rates varied depending on the processing variables evaluated.

1.4.2. Poultry production chain

Cross-contamination during poultry processing plays an important role on the contamination level of the whole production flock processed in the same line. The main processing stages that can enable microbial cross-contamination, inactivation and removal are scalding, defeathering, evisceration, washing and chilling [14•]. Changes in microbial levels in carcasses during these steps are batch- and slaughterhouse-dependent, as the contamination patterns vary between different processing plants [33, 34]. Authors have modelled and quantified the bacterial transfer dynamics at the different stages in the slaughterhouse level. Nauta and others [14•] developed a poultry-processing model that describes the dynamics of cross-contamination between broiler carcasses and the environment (Figure 1.1).

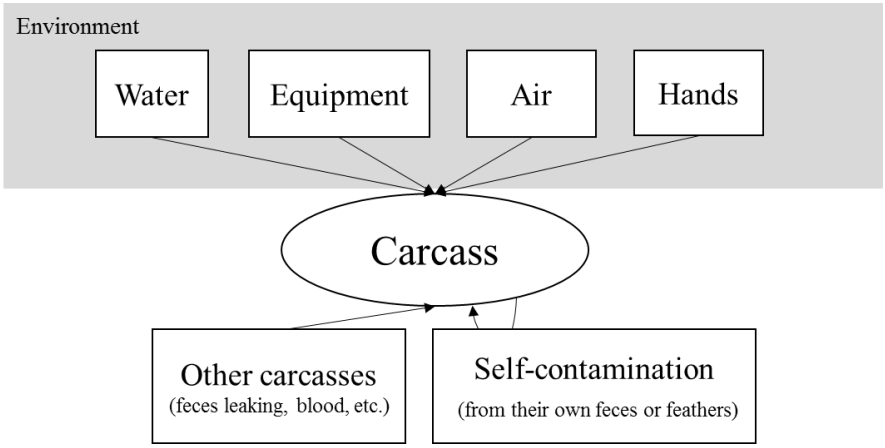

Figure 1.1- Sources of contamination during poultry processing at the slaughterhouse level

Model analysis indicated that cross-contamination is relevant when carcasses entering the processing line present low initial levels of contamination. This is consistent with later findings [35] concluding that the direct route of contamination is more important than cross-contamination in terms of numbers of illnesses at contamination levels equal or above 10^6 cfu. During scalding, inactivation due to the water high temperature and removal processes dominate cross-contamination, as the initial load on carcasses is high and the bacterial transfer from the

environment is relatively low and negligible [14•]. Seliwiorstow and others [36] found out that scalding was the step that less contributed to *Campylobacter* cross-contamination from a contaminated batch to non-contaminated carcasses during the slaughter process, although bacterial contamination can be found in scalding tanks during a processing day [37].

Hayama and others [38] built a simulation model for cross-contamination during poultry processing, assuming that bacterial transfer between individual carcasses is more likely to occur during the defeathering step. Feathers carry high numbers of bacteria which can be transferred directly between carcasses and indirectly via equipment or handling during processing [39]. Additionally, poor handling methods and the high bacterial load in the picker machine make the defeathering step a very important source of cross-contamination [22••, 38].

Evisceration is the step that contributes the most (up to 4 log cfu/g) to *Campylobacter* transfer during the slaughtering process, which can be explained by the spread of fecal material on the equipment [36]. This is also referred as a stage that considerably contributes to the increase in *Salmonella* prevalence on poultry processing line [22••].

The potential risk of cross-contamination during chilling by water immersion can be attributed to the release of organic material and microorganisms from carcasses to water [19•]. If a high number of carcasses enter the chiller at the same time, cross-contamination between carcasses may be a factor considered when developing a model. In addition, some authors consider the water chlorination as an important factor when modelling cross-contamination during chilling. The probability of *C. jejuni* and *S.* Typhimurium contamination in chicken carcasses has been studied [19•] through the development of a predictive model. The authors concluded that the age of chill water and the total chlorine level are important factors to control cross-contamination. However, other authors assumed that cross-contamination does not occur during chilling with water immersion, due to appropriate chlorination [38].

To build cross-contamination models at the slaughterhouse level, assumptions are frequently made and scenarios are setup due to limited data availability or for the sake of simplicity. As cross-contamination patterns vary between processing plants, the application of models developed under certain conditions to other scenarios should be cautious.

1.5. Methods for the evaluation of the robustness of cross-contamination models

Quantitative data of cross-contamination in foods may provide valuable support for understanding the influence of different factors involved in transfer phenomena and in the implementation of intervention measures. However, the use of different methods for sampling and quantification makes the comparison between studies difficult. In this context, more precise, accurate and standardized methods for bacterial quantification during transfer events might contribute to the development of higher performance cross-contamination models to be inserted in Quantitative Microbial Risk Assessments (QMRA), resulting in more reliable risk estimates.

As previously described in this paper, a large number of cross-contamination models are based on empirical approaches since there is still scarce knowledge about the mechanisms ruling bacterial transfer in foods [41]. Some attempts have been recently published to provide more insight on the theoretical basis of transfer phenomena [9•, 19•, 42, 43]. Models' structure can be based on the assumption of different environments where bacteria show different TR. The inherent variability associated to model predictions sometimes do not allow their applicability in QMRA. To this sense, evaluation of model performance is normally used to assess the quality of cross-contamination model predictions.

Available approaches are the use of the acceptable simulation zone (ASZ) [15••, 44], assessment through fitted parameters, QMRA [20] and total transfer potential (TTP).

The use of the ASZ is based on the proportion of relative errors (pRE) (i.e. averaged differences between observed and predicted values) which is a relative measure of model performance because the width of the acceptable prediction zone affects its value [44]. According to the ASZ, a model is found suitable when 70% of counts are inside a predefined acceptable zone around the simulation transfer curve. This approach also offers the advantage that different deviation limits (± log CFU/g) could be tested to calculate the percentage of samples that are satisfactorily predicted. The ASZ was originated as an alternative to bias and accuracy factors [45] demonstrating that the use of pRE could provide an accurate assessment of model performance because pRE considers no growth prediction cases in its calculation of model

performance.

The assessment through fitted parameters can be achieved using standardized residuals [46]. Normality assumption for the error term can be ascertained by testing normality in associated residuals based on normal probability plots. If the assumption is true, residuals should vary randomly around zero and the spread of the residuals should be about the same throughout the plot. One remarkable weakness of the Least Square Regression method is its sensitivity to outliers, which can be also detected by looking at residuals.

Evaluation using QMRA can be done using distributions of microbial prevalence and concentration together with available cross-contamination models in order to estimate the mean risk per serving. Relative risk is also calculated by fitting the cross-contamination model to the data and comparing to a baseline scenario.

Finally, the assessment of TTP is defined by the proportion of bacterial cells in a single sample that is transferred to a whole batch. Actually TTP (%) is a cumulative percentage which assumes that systematic cross-contamination is always present from a contaminated ingredient to a processed food. Although this assumption would lead to cross-contamination overestimation, TTP may be a valid measure for model evaluation [17•].

1.6. Conclusions

The use of compartmental and mechanistic models can be an alternative to further explain the influence of relevant processing factors on cross-contamination dynamics during food processing. However, variability and uncertainty sources around these processes are still high so that parameter estimates obtained by modelling cannot describe bacterial transfer in many cases. The effect of multiple processing, product and environmental factors influences in the occurrence of cross-contamination events. Thus, the application of models developed under certain conditions to other scenarios should be cautious.

In the last years, significant advances have been achieved since individual contribution of factors and corrective measures to be applied (i.e. processing temperatures, equipment material etc.) can be better understood by the application of cross-contamination models. Finally, the use

of alternative performance indices for models evaluation can offer an added value to facilitate their application in food process operations.

1.7. References and recommended readings

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- • Of special interest
- •• Of outstanding interest

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Chapter II

Chapter II: Modelling the inactivation of *Listeria monocytogenes* by high hydrostatic pressure processing in foods: a review

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2.1. Abstract

The application of High Hydrostatic Pressure (HHP) processing technology as a non-thermal pasteurization method has been extensively investigated over the last two decades. *Listeria monocytogenes* is a relevant target for food safety due to its ability to grow and/or survive in a wide range of environmental conditions and be present at hazardous levels in food commodities where lethal treatments have not been carried out, such as some ready-to-eat foods (RTE). This review presents a compilation of modelling studies describing pressure-induced inactivation of *L. monocytogenes* in foods. The influence of a series of factors, including technological parameters, food matrix composition and the physiological state of bacterial cells on inactivation levels is also discussed, as it should be clearly understood and evaluated in order to set and optimize HHP processing conditions. The use of mathematical models to predict the inactivation and probability of recovery of *L. monocytogenes* in foods during HHP application and subsequent storage can help food processors and managers to comply with the current microbiological regulations established for RTE foods, as well as optimize processing conditions.

Keywords: inactivation kinetics, predictive microbiology, mathematical modelling, novel disinfection technique, ready-to-eat food, food safety

2.2. Introduction

Listeria monocytogenes is a psychrotrophic bacteria, considered as a major safety concern in the food industry due to its ability to grow and survive in different types of foods under a wide range of environmental conditions (Das, Lalitha, Joseph, Kamalakanth, & Bindu, 2016). The contamination of ready-to-eat foods (RTE) by *L. monocytogenes* during processing operations such as slicing and packaging is particularly relevant, since these products are generally not submitted to lethal treatments before consumption (Bover-Cid, Belletti, Aymerich, & Garriga, 2015).

The European Commission Regulation No. 2073/2005 requires food operators to demonstrate that RTE foods that support the growth of *L. monocytogenes* do not exceed the limit of 100 cfu/g throughout their shelf-life (European Commission, 2005). In accordance with Codex Alimentarius guidelines, the amount of the pathogen should be limited to 100 cfu/g at the end of shelf life when storage conditions do not permit its growth (Luber, 2011). Otherwise, the absence of *L. monocytogenes* in 25 g of product must be guaranteed (Luber, 2011).

High hydrostatic pressure (HHP) processing is a non-thermal technology that has shown great potential to inactivate pathogenic and spoilage microorganisms, producing microbiologically safer products with extended shelf life and a non-severe impact on the nutritional and organoleptic characteristics of foods (Syed, Buffa, Guamis, & Saldo, 2016). This preservation technique basically consists of the application of isostatic pressures, uniformly and instantaneously transmitted to foods by air-driven pumps through a liquid, generally water (Hugas, Garriga, & Monfort, 2002).

The application of HHP processing has been proposed as a non-thermal pasteurization method to inactivate *L. monocytogenes* in RTE foods (Georget et al., 2015; Syed et al., 2016). Regarding pasteurization of RTE foods with novel technologies, the FDA requires processes that guarantee at least a 5-log reduction of the target microorganism (Saucedo-Reyes, Marco-Celdrán, Pina-Pérez, Rodrigo, & Martínez-López, 2009).

Over the last two decades much effort has been put into process optimization and understanding the inactivation kinetics of *L. monocytogenes* in HHP-processed foods. Mathematical models for predicting inactivation of pathogens constitute useful tools for food processors to select optimum HHP processing conditions (Bover-Cid, Belletti, Garriga, & Aymerich, 2011; Chen, 2007b). Several researchers have highlighted the need for databases containing kinetic model parameters for target microorganisms (Valdramidis, Taoukis, Stoforos, & Van Impe, 2012). Furthermore, predictive models can help food industries to comply with process criteria and current regulations for *L. monocytogenes* in RTE foods (Hereu, Dalgaard, Garriga, Aymerich, & Bover-Cid, 2012).

Microbial inactivation through the application of HHP processing has been modelled as a function of technological processing variables and food intrinsic factors/characteristics. This review presents a compilation of inactivation models of *L. monocytogenes* during HHP processing of foods, as well as logistic models of its behaviour during storage. First, an overview of inactivation kinetic models is presented, followed by a discussion on the different factors influencing the inactivation of *L. monocytogenes* induced by the application of HHP technology, which include technological parameters, food matrix characteristics and culture conditions.

2.3. *Listeria monocytogenes* inactivation kinetics by HHP

Various predictive models are available for HHP inactivation of *L. monocytogenes* or *Listeria innocua* (a *L. monocytogenes* surrogate for processing plant safety purposes) in food simulated systems (Ates, Rode, Skipnes, & Lekang, 2016; Doona, Feeherry, Ross, & Kustin, 2012), meat products (Bover-Cid et al., 2015, 2011; Carlez, Rosec, Richard, & Cheftel, 1993; Hereu, Dalgaard, et al., 2012; Lerasle et al., 2014; Rubio, Possas, Rincón, García-Gímeno, & Martínez, 2018), fish (Ramaswamy, Zaman, & Smith, 2008), seafood (Das et al., 2016; Fletcher, Youssef, & Sravani, 2008), milk (Amina, Kodogiannis, Petrounias, Lygouras, & Nychas, 2012; Buzrul, Alpas, Largeteau, & Demazeau, 2008; Chen & Hoover, 2003, 2004), dairy products (Shao,

Ramaswamy, & Zhu, 2007) and RTE vegetables (Jung, Lee, Kim, Cho, & Ahn, 2014; Muñoz, Ancos, Sa, & Cano 2006).

Although bacterial resistance to HHP has been reported to be higher in solid foods than in culture media and liquid foods (Ates et al., 2016; Bover-Cid et al., 2015), a substantial number of modelling approaches developed in buffered solution and culture media is available in literature (Muñoz-Cuevas et al., 2013). Despite some limitations, the development of predictive models in model systems may offer certain advantages, such as high reproducibility, rigorous control of environmental factors and the absence of interfering background microbiota, but, prior to application, their validation on target foods is highly recommended (Baka, Noriega, Van Langendonck, & Van Impe, 2016).

Pressure inactivation models can be classified into primary, secondary and tertiary models in the same way as traditional predictive models (Whiting & Buchanan, 1993).

2.3.1. Primary models

Primary models in HHP technology are mathematical equations describing changes in microbial counts induced by pressure as a function of treatment times. The most frequently applied equations are described in sections 2.3.1.1 and 2.3.1.2. These models are useful when evaluating the inactivation of *L. monocytogenes* at fixed conditions, such as at a specific temperature and pressure level.

2.3.1.1. Linear models

Studies have shown that the pressure destruction kinetics of *L. monocytogenes* in foods as a function of pressure-holding times may follow a first order process in which the number of viable cells inactivated decreases proportionally depending on treatment time (Phua & Davey, 2007). This relationship is represented in Equation 2.1. Studies where *L. monocytogenes* behaviour followed a linear trend during high pressure treatments are shown in Table 2.1.

$$log\left({N}/{N_0}\right) = -kt = -\frac{t}{D_P} \qquad \text{Eq. (2.1)}$$

where, assuming static conditions of pressure and temperature, N refers to the number of survivals in samples after pressure treatments; N_0 is the number of viable cells just before application of a pressure level set in the experimental design; t is the pressure-holding time; k is the inactivation rate constant of bacteria number at pressure P due to HPP treatments; and D_P is the time required for one log reduction of bacteria number due to HPP treatments.

2.3.1.2. Non-linear models

Despite the increasing number of published studies in which linear inactivation kinetics have been observed, patterns of microbial inactivation during HHP are frequently non-linear. Non-linear behaviour during pressure treatments is attributed to cumulative damage to microbial cells, which simultaneously affects a combination of processes or functions (Tay, Shellhammer, Yousef, & Chism, 2003).

The non-linear functions most commonly applied to describe *L. monocytogenes* inactivation kinetics under HHP are the Weibull model, the log-logistic function, the modified Gompertz equation and the Baranyi model (Table 2.2). Although these sigmoidal functions were originally developed for fitting growth curves, they have been restructured by authors and used to describe microbial survival curves after thermal treatments (Cole, Davies, Munro, Holyoak, & Kilsby, 1993; Linton, Carter, Pierson, & Hackney, 1995).

A tail-shaped pattern is frequent in non-linear inactivation models (Buzrul & Alpas, 2004; Hereu, Dalgaard, et al., 2012; Muñoz-Cuevas et al., 2013). The most accepted hypothesis to explain the tailing effect is the presence of subpopulations within a microbial population that are more resistant to pressure treatments and remain viable even after prolonged pressure holding times (Gayán, Torres, & Paredes-Sabja, 2012). The presence of a shoulder on inactivation curves has also been reported, characterized by a low rate of cell inactivation at the beginning of pressure treatments (Doona et al., 2012; Fletcher et al., 2008). Some hypotheses have been put forward to explain the shoulder in inactivation curves, such as non-uniform delivery of pressure into the product and different pressure sensitivities of the target microorganism (Bermúdez-

Aguirre & Barbosa-Cánovas, 2011). The mechanism of tailing and shouldering needs to be elucidated in future studies to enable effective pressure treatments to be established.

Among the non-linear models, the Weibull model is the one most popularly applied to describe HHP-induced inactivation, due to its flexibility and simplicity (Bermúdez-Aguirre & Barbosa-Cánovas, 2011; Serment-Moreno, Barbosa-Cánovas, Torres, & Welti-Chanes, 2014). The Weibull distribution in pressure-induced inactivation events can be interpreted as a cumulative function that determines the exposure time at which the bacterial cells fail to resist pressures and become inactivated. This distribution assumes that the resistance of microorganisms present in a population differs from cell to cell (Chen & Hoover, 2004; Serment-Moreno et al., 2014).

Drawbacks have been reported in the application of the Weibull model to describe microbial inactivation by HHP, such as the fact that its parameters (n = shape parameter and δ = scale parameter) are dependent or strongly correlated, leading to instability in their estimations (Buzrul, Alpas, et al., 2008; Chen & Hoover, 2004; Doona et al., 2012). Mafart, Couvert, Gaillard, and Leguerinel (2002) found it worthwhile to fix the n parameter value at a probability-averaged characteristic of a strain, thereby enabling the δ values to be estimated from a linear regression. This leads to better stability of the δ values and increases the robustness of the model (Couvert, Gaillard, Savy, Mafart, & Leguérinel, 2005). Many authors have followed this procedure of fixing the n-value to obtain δ parameter estimates (Buzrul & Alpas, 2004; Chen & Hoover, 2004; Lerasle et al., 2014).

The log-logistic model assumes that bacterial cells in a population have different pressure resistances and that these differences are permanent (Chen, 2007b). Chen, Joerger, Kingsley, and Hoover (2004), Chen & Hoover (2003) and Muñoz-Cuevas et al. (2013) compared the application of the Weibull, log-logistic and Gompertz models to fit the same sets of pressure inactivation data. The Gompertz model was the one that gave the poorest fits in all cases, with the exception of the study by Muñoz-Cuevas et al. (2013) in which the Weibull did not accurately describe the experimental data. Studies describing primary non-linear models of *L. monocytogenes* inactivation kinetics in foods are listed in Table 2.3.

The Baranyi model has also shown better prediction performance and more robustness than the modified Gompertz equation when fitted to survival and inactivation curves (Saucedo-Reyes et al., 2009; Xiong, Edmondson, Linton, & Sheard, 1999). According to Xiong et al. (1999), the Baranyi model can fit curves of the four most common shapes (i.e. linear, linear with shoulder, linear with tail and linear with both shoulder and tail) and one of the advantages of its use in comparison with the modified Gompertz equation is its capacity to indicate to a certain extent if the tailing phase in a survival curve is significant.

In addition to the most common statistical goodness-of-fitness indexes usually calculated to evaluate model performance (RMSE, R^2, adjusted R^2), simplicity is an important factor when a model is selected. A model with more parameters is expected to show a better fit to data. However, over-parameterization can result in equations that describe not only the underlying response, but also observed data errors (Baranyi, Ross, McMeekin, & Roberts, 1996; Chen & Hoover, 2003). Considering the number of parameters of the models described, the Weibull model would be the simplest one to apply to describe the non-linear inactivation kinetics of *L. monocytogenes* in foods. This model is also the most versatile as it can accurately fit concave upwards, concave downwards or straight (n = 1) survival curves (Mañas & Pagán, 2005).

2.3.2. Secondary models

Secondary modelling is applied to predict changes in the kinetics parameters of primary inactivation models as functions of intrinsic or extrinsic factors.

The pressure or temperature dependence of the inactivation rate constant (k= 2.303/D_P-values) is frequently analysed by the Arrhenius-type model and Pressure Death Time model (Mussa, Ramaswamy, & Smith, 1998, 1999; Ramaswamy et al., 2008; Shao et al., 2007). In the Arrhenius-type model, it is possible to analyse the pressure sensitivity of k by plotting ln k values versus pressure. Estimation of the volume change in inactivation ($\Delta V^{\neq}$), which is a measure of the net effect of pressure on reactions causing physiological change at constant temperature, can be based on the slope of the "ln k versus pressure" regression (Equation 2.2).

$$\Delta V^{\neq} = -RT\left[\frac{\Delta(lnk)}{\Delta P}\right] \qquad \text{Eq. (2.2)}$$

where P is the pressure (MPa); k is the rate constant (1/min); T is the absolute temperature (K); R is the gas constant (8.314 x 10^{-6} m^3/mole MPa/K); and $\Delta V^{\neq}$ is the volume change in activation (m^3/mole) .

In the Pressure Death Time model, sensitivity of the D_P-values to changes in pressure can be expressed as Z_P-values, which is the pressure range increase for one log cycle (tenfold) decrease in the D_P-value (Equation 2.3). Various Z_P-values determined in different food matrices are shown in Table 2.1.

$$Z_P = (P_2 - P_1) / Log(D_{P2}/D_{P1}) \quad \text{Eq. (2.3)}$$

The Z_P-values can also be estimated when inactivation kinetics follow a non-linear trend. This is the negative reciprocal slope of the regression resultant from plotting the log δ values estimated from the Weibull model (Chen & Hoover, 2004; Lerasle et al., 2014). For the Gompertz and Baranyi models, Z_P-values were determined as the inverse value of the gradient for the linear relation between the decimal logarithms of the primary parameters (μ_{max} and $k_{max,}$ Table 2.2) and the pressure applied (Saucedo-Reyes et al., 2009).

2.3.3. Tertiary models

Tertiary modelling consists on the incorporation of predictive models on application programs such as computer software tools (Buchanan, 1993). The incorporation of predictive models on user-friendly applications enables food processors and managers to assess the influence of a series of factors on the effectiveness of HHP inactivation treatments without complexity and in a quick manner. An example of an application tool that can be used to assess the microbial inactivation in foods that have undergone high-pressure processing is the one named "HP3". This tool was developed at IRTA (Institute of Agrifood Research and Technology, Monels, Spain) and is available at http://www.hp3.cat/.

Table 2.1- Studies in which *L. monocytogenes* inactivation in foods followed a first-order kinetic process at different pressures and respective inactivation parameters

Food systems	***Technological parameters*** ***Pressure MPa***	***Pressure-holding time min***	***Temperature °C***	***D_P-values[1](Pressures) min (MPa)***	***Z_P-values[2] MPa***	***Reference***
Minced beef muscle	50-400	0-20	4-50	6.5 (330), 5.0 (360)	NR	Carlez et al. (1993)
Fresh pork chops	200-400	0-90	25	63.1 (200), 30.8 (250), 16.2 (300), 8.52 (350), 3.52 (400)	163	Mussa et al. (1999)
Fish slurry	250-400	0-60	20-25	40.1 (250), 14.7 (300), 4.16 (350), 1.49 (400)	103	Ramaswamy et al. (2008)
White prawn muscle	250-400	0-40	30	34.52 (250), 11.81 (300), 5.92 (350), 5.1 (400)	NR	Das et al. (2016)
Milk	150-400	0-120	25	84.4 (150), 46 (250), 26.6 (300), 13.9 (350)	266	Mussa et al. (1998)
	300-600	1-105	25	10.99 (300), 6.00 (400), 2.43 (600)	480	Dogan and Erkmen (2004)
	300-500	< 10	30	9.56 (300)	NR	Xu et al. (2009)
	400	1-30	20-25	0.23 (400)	NR	Hayman et al. (2007)
Raw milk cheese	250-350	0-45	25	23.5 (250), 3.6 (300), 1.4 (350)	82	Shao et al. (2007)
Mango juice	250-550	0-60	20-25	13.6 (250), 5.23 (300), 2.01 (350), 0.80 (400)	121	Hiremath and Ramaswamy (2012)
Peach juice	300-600	1-70	25	6.17 (300), 3.39 (400), 1.52 (600)	506	Dogan and Erkmen (2004)
Orange juice	300-600	1-30	25	2.87 (300), 1.80 (400), 0.87 (600)	576	Dogan and Erkmen (2004)

Food systems	***Technological parameters***			***D_P-values[1](Pressures)***	***Z_P-values[2]***	***Reference***
	Pressure MPa	***Pressure-holding time min***	***Temperature °C***	***min (MPa)***	***MPa***	
	300-500	< 10	30	1.11 (300)	NR	Xu et al. (2009)
Tomato juice	300-500	< 10	30	0.94 (300)	NR	Xu et al. (2009)
Brain heart infusion broth	200-700	1-95	25	12.11 (200), 8.22 (300), 3.79 (400), 2.62 (500), 1.63 (600), 1.14 (700)	578	Dogan and Erkmen (2004)
Peptone solution	137.9-344.7	5-15	25-50	50.8 (137.9), 35.2 (206.8), 21.6 (275.8), 14.3 (344.7)	368.7	Alpas et al. (1998)
Saline solution	400	0-9	24	NR	NR	Mohamed, Diono, and Yousef (2012)

[1]D_P values estimated at room temperature (20-25 °C) in the studies where a range of temperatures was evaluated
[2]Z_P-value = the pressure range increase for one log cycle (tenfold) decrease in the D_P-value
[3]NR = Not reported

Table 2.2- Most used non-linear primary functions to model the effect of pressure levels on *L. monocytogenes* inactivation kinetics

Function	***Equation***	***Parameters***	***Reference***
Weibull	$log\left(N/N_0\right) = -bt^n = -(\frac{t}{\delta})^n$	δ = scale parameter n = shape parameter	Peleg and Cole (1998)
log-logistic	$log\left(N/N_0\right) = \frac{A}{1 + e^{4\sigma(\tau - logt)/A}} - \frac{A}{1 + e^{4\sigma(\tau+6)/A}}$	A = the difference in value of the lower and upper asymptotes σ = maximum rate of inactivation τ = log time to the maximum rate of inactivation	Cole et al. (1993)
Baranyi	$log\left(N/N_0\right) = Log(q_B + (1 - q_B)e^{-k_{max}(t - B(t))}$ $B(t) = \frac{r}{3}(\frac{1}{2}ln\frac{(r+t)^2}{r^2 + rt - t^2} + \sqrt{3}arctan\frac{2t - r}{r\sqrt{3}} + \sqrt{3}arctan\frac{1}{\sqrt{3}}$	k_{max} = maximum death rate r = time required for the relative death rate to reach half of k_{max} q_B = tailing rate	Baranyi and Roberts (1994)
Gompertz	$log\left(N/N_0\right) = Ce^{-e^{BM}} - Ce^{-e^{-B(t-M)}}$	M= time at which the death rate is maximum B = relative death rate at M C = the difference in value of the upper and lower asymptotes	Gibson, Bratchell, and Roberts (1988)

[1] N refers to the number of survivals in samples after pressure treatments; N_0 is the number of viable cells just before pressures achieved the intensities set in the experimental design; t is the exposure time.

Table 2.3- Studies describing primary non-linear models of *L. monocytogenes* inactivation kinetics in foods

Food system	*Technological parameters* *Pressure (MPa)*	*Pressure-holding time (min)*	*Temperature (ºC)*	*Primary non-linear model*	*Reference*
Poultry meat	200-500	2-14	15	Weibull	Lerasle et al. (2014)
Turkey breast meat	300-500	1-2	1-55	Weibull	Chen (2007b)
Ready-to-eat meats	300-800	0-15	15	log-linear with tail	Hereu, Dalgaard, et al. (2012)
Whole milk	600	0-30	21.5	log-logistic and Weibull	Chen (2007a)
	400-600	0-180	20-25	Weibull	Chen and Hoover (2004)
	400-600	0-80	22	Weibull	Buzrul, Alpas, et al. (2008)
	400-600	0-30	27-60	Weibull	Mishra et al. (2013)
	400 and 500	0-120	22-50	log-logistic and Weibull	Chen and Hoover (2003)
Tryptone soya broth	325-400	0-20	25	Gompertz and Baranyi	Saucedo-Reyes et al. (2009)
	350-450	0-23	25	log-logistic and Gompertz	Muñoz-Cuevas et al. (2013)
Peptone solution	138-345	0-30	25-50	log-logistic and Weibull	Buzrul and Alpas (2004)
Deionized water	200-400	0-20	25	Weibull	Gou et al. (2010)
Yogurt drink	600	0-5	25	log-logistic and Weibull	Evrendileck and Balasubramaniam (2011)
Ready-to-eat vegetables	500	0-30	20	Weibull	Jung et al. (2014)

2.3.4. Other modelling structures

In this section, polynomial models describing the inactivation of *L. monocytogenes* during food processing by HHP depending on technological parameters (pressure, temperature, pressure-holding times), and intrinsic factors or food characteristics (pH, a_w, fat and salt content, etc.) are discussed and cited. Logistic approaches that take into consideration the recovery of pressure-injured cells are also described.

2.3.4.1. Polynomial models

Polynomial functions take into account the effect of individual factors on inactivation parameters or the interaction between them, as follows:

$$log\left({N}/{N_0}\right) = B_0 + \sum_{i=1}^{n} B_i\, x_i + \sum_{i=j=1}^{n} B_{ij} x_i x_j \qquad \text{Eq. (2.4)}$$

where B_0 is a constant; B_i-B_{nn} are model coefficients and x_i-x_n are the input variables.

The empirical technique known as Response Surface Methodology (RSM) is commonly used to process optimization and to model the influence of a set of factors in *L. monocytogenes* pressure-induced inactivation in culture media or food systems. Polynomial models generated through RSM are shown in Table 2.4. RSM methods (Central Composite Design, Box-Behnken design, etc.) can give the optimal fitting of polynomial models from a minimal number of experiments and enable study of the interaction between factors on the response of interest, including nonlinearities on curves (Alfaia et al., 2015; Ates et al., 2016; Chien, Sheen, Sommers, & Sheen, 2016). However, Koseki and Yamamoto (2007a) highlight the harmful use of polynomial models as they may include terms without biological meaning. Besides, extrapolation must be avoided when using purely empirical models such as RSM models, as their application outside the domain of the data used for parameters estimation could yield nonsensical results.

Table 2.4- Polynomial models describing the inactivation/survival of *L. monocytogenes* in food systems as a function of HHP technological parameters and food composition/characteristics

Food system	*Independent variables (range)*	*Model*	*Adj R^2*	*Reference*
Dry cured ham	Pressure (*P*) (347-852 MPa) Pressure-holding time (*t*) (2.3-15.75 min)	$log\left(N/N_0\right) = -380.3164 + 292.5942 \cdot logP - 56.1268 \cdot (logP)^2 + 1.4090 \cdot t + 0.0133 \cdot t^2 - 0.6423 \cdot logP \cdot t$	0.99	Bover-Cid et al. (2011)
Dry cured ham	Pressure (*P*) (347-852 MPa) a_w (0.86-0.96) Fat content (*F*) (10-50 %)	$log\left(N/N_0\right) = 38.653 - 34.29 \cdot a_w - 0.0237 \cdot P - 0.00349 \cdot F^2 + 3.34 \cdot 10^{-4} \cdot P \cdot F$	0.84	Bover-Cid et al. (2015)
Spanish chorizo sausage	Pressure (*P*) (349-600 MPa) Pressure-holding time (*t*) (0-12.53 min) a_w (0.79- 0.92)	$log\left(N/N_0\right) = -176.7705 + 394.9484 \cdot a_w - 237.7673 \cdot a_w^2 + 0.0610 \cdot P - 0.0001 \cdot P^2 + 0.5461 \cdot t - 0.02839 \cdot t^2 - 0.0008 \cdot P \cdot t$	0.88	Rubio et al. (2018)
Buffered solution	pH (4-10) Soybean protein (*Pr*) (0- 5 %) Sucrose (*S*) (0.25-13.25 %)	$log\left(N/N_0\right) = -11.1234 + 1.3667 \cdot pH + 0.3789 \cdot Pr - 0.0845 \cdot S - 0.1235 \cdot pH^2 + 0.0416 \cdot pH \cdot S - 0.0404 \cdot Pr \cdot S$	0.84	Gao et al. (2007)
Peptone solution	Pressure (*P*) (207-345 MPa) Pressure-holding time (*t*) (10-30 min) Temperature (*T*) (25-45 ºC)	$log\left(N/N_0\right) = -25.02 + 0.1217 \cdot P + 0.111 \cdot t + 0.582 \cdot T - 1.407 \cdot 10^{-4} \cdot P^2 + 2.5 \cdot 10^{-3} \cdot t^2 - 2.8 \cdot 10^{-3} \cdot T^2 - 5.507 \cdot 10^{-4} \cdot P \cdot t - 1.406 \cdot 10^{-3} \cdot P \cdot T - 4.10^{-3} \cdot t \cdot T$	0.85	Buzrul, Çevik, et al. (2008)
Milk buffer	Pressure (*P*) (232-568 MPa) Pressure-holding time (*t*) (1.6-18.4 min) Temperature (*T*) (23-57 ºC)	$log\left(N/N_0\right) = +2.359 - 4.17 \cdot 10^{-3} \cdot P + 0.0371 \cdot t - 0.363 \cdot T - 1.12 \cdot 10^{-5} P^2 - 1.34 \cdot 10^{-3} \cdot t^2 + 0.01128 \cdot T^2$	0.98	Gao, Ju, and Jiang (2006)
Model soup	Pressure (*P*) (359-641 MPa) Temperature (*T*) (15.9-44.1 ºC)	$log\left(N/N_0\right) = 20.67 - 0.06186 \cdot P - 0.0993 \cdot T + 3.56 \cdot 10^{-5} \cdot P^2$	0.95	Ates et al. (2016)

Food system	*Independent variables (range)*	*Model*	*Adj R^2*	*Reference*
Bean sprouts juice	Pressure (*P*) (150-400 MPa) Temperature (*T*) (20-40 ºC)	$log(^{N}/_{N_0}) = -0.97 + 6.4 \cdot 10^{-3} \cdot P + 0.024 \cdot T - 2.1 \cdot 10^{-5} \cdot P^2 - 8.2 \cdot 10^{-4} \cdot T^2 + 2.8 \cdot 10^{-5} \cdot P \cdot T$	0.90	Muñoz et al. (2006)
Smoothies	Pressure (*P*) (100-300 MPa) Temperature (*T*) (-5-45 °C)	$log\ N = 5.782 - 0.012 \cdot P + 0.001 \cdot T \cdot P - 2.764 \cdot 10^{-5} \cdot T^2 P - 1.468 \cdot 10^{-6} \cdot T \cdot P^2 + 4.218 \cdot 10^{-8} \cdot T^2 \cdot P^2$	0.99	Scolari et al. (2015)

[1] *N* refers to the number of survivals in samples after pressure treatments; N_0 is the number of viable cells just before pressures achieved the intensities set in the experimental design.

2.3.4.2. Logistic models

At the boundary between survival and death, the modelling approach needs to shift from a kinetic to a logistic model that considers the recovery of pressure-injured cells after treatments. The output of logistic models is the chance of recovery of bacteria cells during food storage or shelf life after exposure to HHP treatments.

Logistic regression is used to relate input variables with a dependent variable expressed as binary response (0, 1). Input variables are generally technological parameters or food characteristics. The logit function is defined by Equation 2.5.

$$logitPr = Ln(\frac{Pr}{(1-Pr)}) \quad \text{Eq. (2.5)}$$

where *Pr* is the probability of the outcome of interest.

Logistic models of HHP-induced microbial inactivation (Table 2.5) are more realistic approaches as they consider the recovery of injured cells during storage of processed foods, while kinetic inactivation models are based on survival data obtained immediately after the experiments and do not take the recovery of pressure-injured cells into consideration.

Table 2.5- Logistic modelling approaches of the recovery of *L. monocytogenes* cells following HHP treatments

Food systems	***Independent variables (range)***	***Model***	***Reference***
Meat simulation medium	Pressure (P) (450-800 MPa) a_w (0.955-0.987) Storage time (t_s) (0-28 d)	$logit(P) = 62.08 - 1.83 \cdot 10^{-1} \cdot P + 1.38 \cdot 10^{-4} \cdot P^2 - 0.18 \cdot 10^{-3} \cdot P \cdot t_s - 4.25 \cdot 10^{-3} \cdot P \cdot a_W$	Valdramidis et al. (2015)
Sliced cooked ham	Pressure (P) (400-600 MPa) Pressure-holding time (t) (1-60 min) Storage time (t_s) (0-70 d) Inoculum level (IC) (3 or 5 log cfu/g)	$logit(P) = 16.8423 - 0.0722 \cdot P - 7.889 \cdot Log(t) + 0.1508 \cdot t_s + 4.9037 \cdot IC$	Koseki, Mizuno, and Yamamoto (2007)
Phosphate buffer solution	Pressure (P) (200-500 MPa) Pressure-holding time (t) (1-30 min) pH (3-7) Inoculum level (IC) (3-7 log cfu/mL)	$logit(P) = 12.9973 - 0.0775 \cdot P - 9.1909 \cdot Log(t) + 2.3331 \cdot pH + 1.6674 \cdot IC$	Koseki and Yamamoto (2007a)

2.4. Modelling the effect of technological factors on HHP-induced inactivation

The most important technological factors influencing the inactivation of *L. monocytogenes* by HHP are pressure, pressure holding-time and temperature. In general, by increasing the pressure level and pressure-holding time, an increase in the lethal effect of HHP treatments is achieved (Bover-Cid et al., 2015; Buzrul & Alpas, 2004; Hereu, Dalgaard, et al., 2012; Juck, Neetoo, Beswick, & Chen, 2012; Muñoz-Cuevas et al., 2013; Youart, Huang, Stewart, Kalinowski, & Legan, 2010). The main consequence of high pressure application on microbial cells is damage to membranes, leading to leakage of cell content and the destruction of vital complexes (Bowman, Bittencourt, & Ross, 2008; Ferreira, Almeida, Delgadillo, Saraiva, & Cunha, 2016).

Mussa et al. (1999) conducted one of the first modelling studies of *L. monocytogenes* inactivation by HHP in meats, obtaining kinetic data on samples of pork chops. The independent variables studied were pressure intensities (200-400 MPa) and the duration of pressure treatments (0-90 min). Pressure inactivation kinetics were observed to follow a first-order kinetic process, with D_P decreasing from 63.1 min at 200 MPa to 3.52 min at 400 MPa. Table 2.1 shows the decrease in D_P-values with increases in pressure reported in various studies.

In the study by Bover-Cid et al. (2011), pressure and pressure-holding time were the most important factors influencing microbial inactivation induced by HHP on dry-cured ham. These authors used the RSM following a Central Composite Design to develop a polynomial model for *L. monocytogenes* inactivation as a function of pressure intensities, pressure-holding time and fluid temperature. An interaction term on the polynomial model relating pressure and time indicates the synergistic effect between these two variables on inactivating *L. monocytogenes*. The synergistic effect between pressure and time was also highlighted in other published models (Buzrul, Çevik, et al., 2008; Scolari, Zacconi, Busconi, & Lambri, 2015) (Table 2.4). Also, with respect to the study by Bover-Cid et al. (2011), an increase in holding time for longer than 10 min did not lead to a meaningful increase in inactivation, and little lethal effect was observed when pressures below 450 MPa were applied. Accordingly, at moderate pressure ranges (< 450 MPa) an increase in pressure-holding times did not significantly enhance cell inactivation in

tryptone soya broth (TSB) (Alpas, Kalchayanand, Bozoglu, & Ray, 1998; Muñoz-Cuevas et al., 2013).

Many potential HHP applications would require long treatment times at high pressure intensities to ensure an adequate inactivation level of pathogens and spoilage microorganisms. Otherwise, pressure treatments alone would not be sufficient to guarantee food safety (Das et al., 2016). Besides, long pressurization treatments are not economically viable in the food industry. One alternative for decreasing the intensity and duration of pressure treatments is the combination of HHP with mild heat treatments (Chen & Hoover, 2003).

The fluid temperature, also referred to as the process or treatment temperature, is a technological parameter that plays an important role in the inactivation kinetics of microorganisms during HHP treatments. Although the fluid temperature was not a significant factor influencing *L. monocytogenes* inactivation in the range considered by Bover-Cid et al. (2011) for model development (7.6 - 24.4 °C), other studies reported an increase in bacterial inactivation with the increase in process temperature (Alpas et al., 2000; Juck et al., 2012; Kalchayanand, Sikes, Dunne, & Ray, 1998; Mishra, Puri, & Demirci, 2013; Syed et al., 2016). Juck et al. (2012) reported that the increase in inactivation is usually enhanced at temperatures above room temperature, which is out of the temperature range evaluated in the study by Bover-Cid et al. (2011). The effect of temperature is corroborated by Fletcher et al. (2008), who modelled the log-linear inactivation rates of *L. monocytogenes* in mussel meat as a function of process temperature during pressure treatments at 400 MPa. The D_T-values, defined in their study as the time to achieve one log reduction at a given temperature, decreased from 3.94 min at 11°C to 0.315 min at 40 °C, indicating that the increase in temperature enhanced inactivation at a constant pressure level. Accordingly, the increase in process temperature values ranging from -17 to 32 °C potentiated the listericidal effect of HHP on sliced cooked ham at 500 MPa (Teixeira, Maier, Miller, Gänzle, & McMullen, 2016).

Low fluid temperatures can also enhance the effectiveness of high pressures on *L. monocytogenes*. Chen (2007a) observed that outside the temperature range between 10 and 30 °C, the sensitivity of *L. monocytogenes* to pressure treatments increased considerably in turkey

breast meat at 220 MPa, with D_T-values equal to 10.1, 25.9, 39.0, 15.5, 10.1, and 1.9 min at −1, 10, 20, 30, 40, and 45 °C, respectively. Pressure-induced inactivation of *L. monocytogenes* was also potentiated by low (-5 °C) and high temperatures (45 °C) in smoothies (Scolari et al., 2015).

The combination of high pressures with mild heat treatments by controlling fluid temperature can be considered a hurdle technology for inactivating microorganisms in foods. As reported in this section, a combination of lower pressure levels with elevated or low fluid temperature could be used instead of higher pressures and medium temperatures, thereby reducing the operating costs of HHP processing technology. However, the effects of elevated temperatures on the organoleptic characteristics of foods must be taken into consideration, as research has revealed that, depending on the processing temperature and other technological parameters, HHP leads to undesirable changes in food quality (Sun & Holley, 2010).

2.5. Modelling the effect of the food matrix on HHP-induced inactivation

Besides technological parameters, food components, i.e. fats, proteins, sugars, minerals, food intrinsic factors (pH, a_w) and additives, can interfere with the effectiveness of HHP in inactivating bacteria (Bover-Cid et al., 2015; Syed et al., 2016).

2.5.1. Food components

The influence of fat content on HHP-induced inactivation is not well elucidated and published studies are controversial (Bover-Cid et al., 2015). Some studies have revealed that the increase in fat content results in an increase in the pressure resistance of microorganisms to HHP (Syed et al., 2016). On the other hand, the fat content was not a significant factor influencing *L. monocytogenes* pressure-induced inactivation in a simulated food system (Gao, Ju, & Wu-Ding, 2007) and in minced chicken (Escriu & Mor-Mur, 2009).

Results obtained by Hereu, Dalgaard, et al. (2012) indicated that the fat content of mortadella (~ 17 %) would exert a protective effect from pressure on *L. monocytogenes* cells, in comparison with cooked ham, which has a lower fat content (~ 4.5 %). The inactivation kinetics of *L.*

monocytogenes in both products could not be described by a general model when pressures ranging from 300 to 800 MPa were applied during 0-15 min.

Bover-Cid et al. (2015) concluded that the level of fat affecting the process lethality of HHP was dependent on the pressure intensities. The authors generated a polynomial model describing the effect of fat content and pressures on the inactivation of *L. monocytogenes* by HHP in dry-cured ham during 5 min at 15°C. At pressures lower than 650 MPa, fat content and inactivation were directly proportional, while lower inactivation levels were recorded at pressures higher than 650 MPa, attributable to the fat protective effect. Interestingly, a term indicating an interaction between fat content and pressure in the polynomial model, represented in Figure 2.1a, shows that food composition and technological parameters can act synergistically on the effectiveness of HHP.

Food proteins seem to protect microorganisms during pressure treatments, with higher microbial resistance on proteinaceous medium in comparison with buffer medium (Gao et al., 2007). For instance, pressure treatments on milk were not so effective as on brain heart infusion broth, which was attributed to the protective effect of higher protein and fat levels present in milk (Dogan & Erkmen, 2004).

The protective effect of the food matrix carbohydrate content (glucose, fructose, galactose and sucrose) during HHP may be associated with different mechanisms: the reduction of a_w with protein and membrane stabilization and the properties of sugar that can act as kosmotropic solutes, thereby increasing system stability and providing additional protection to pressures (Gao et al., 2007; Georget et al., 2015).

Gao et al. (2007) developed a polynomial model of *L. monocytogenes* inactivation during HHP processing at 448 MPa for 11 min at 41 °C (Table 2.4). Food matrix samples based on a physiological solution were prepared with different pH concentrations of soybean protein, sucrose and bean oil. Among these four independent variables considered for model development, the bean oil concentration was the only one that had no influence on *L. monocytogenes* inactivation. Protein and sucrose exert a synergistic effect on protecting *L.*

monocytogenes during pressurization, with a reduction in treatment effectiveness at increasing concentrations (represented in Figure 2.1d).

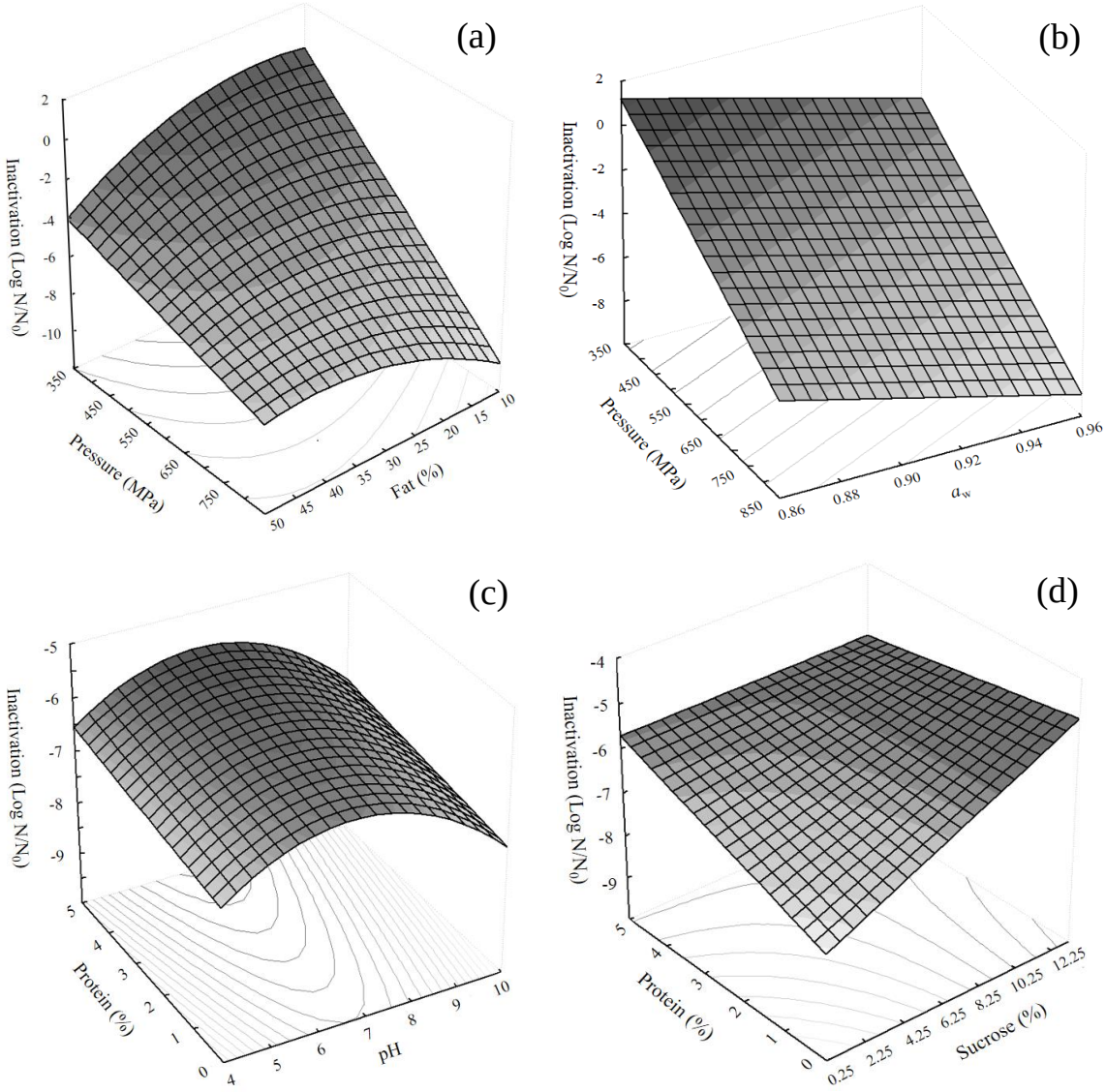

Figure 2.1- Effect of different factors on the inactivation of *L. monocytogenes* by high hydrostatic pressure. Graphs (a) and (b) were constructed based on the model of Bover-Cid et al. (2015) in dry-cured ham. Graphs (c) and (d) were constructed based on the model of Gao et al. (2007) in a food model. Model equations are available in Table 2.4

The influence of food composition on the inactivation of *L. monocytogenes* is complex, making it difficult to address the effect of each individual component on pressure-induced inactivation. Pressure lethality seems to be related simultaneously to both food composition and

technological parameters and to factors that act synergistically. This evidences the need for product-oriented approaches when evaluating HHP processing to inactivate bacteria, taking into consideration the specific characteristics of a food product in order to set HHP processing conditions.

2.5.2. pH and a_w

Inactivation of *L. monocytogenes* by HHP in foods can be conditioned by the food matrix intrinsic factors pH and a_w. In general, microorganisms are more susceptible to pressure in foods with lower pH and suspensions (Ferreira et al., 2016; Koseki & Yamamoto, 2007a).

Low pH resulted in a noticeable synergistic effect with pressure on the inactivation of *L. monocytogenes* in orange (pH = 3.46) and tomato juices (pH = 4.11) in comparison with deionized water and milk, with pH values of 6.76 and 6.85, respectively (Xu, Hyeon-Yong, & Ahn, 2009). Dogan and Erkmen (2004) also concluded that inactivation was enhanced by a decrease in pH values when evaluating the inactivation kinetics of *L. monocytogenes* in liquids at 600 MPa having D_P-values equal to 2.43, 1.52 and 0.87 min in milk (pH = 6.64), peach juice (pH = 5.21) and orange juice (pH = 3.55), respectively.

Likewise, Gao et al. (2007) found that an increase in pH from 4 to 6.6 led to a decrease in inactivation levels, while increasing pH from 6.6 to 10 resulted in increased inactivation levels. Thus, the combination of high and low pH values with HHP processing resulted in a higher efficacy of this technology for reducing *L. monocytogenes* levels. On the other hand, at alkaline pH food proteins can be denaturized, thus the protective effect of protein on the target microorganism is reduced and, consequently, higher inactivation levels must be attained at higher pH values. Their polynomial model also revealed an interactive effect between pH and protein levels on *L. monocytogenes* inactivation (Figure 2.1c).

Regarding the effect of a_w, there is a marked increase in HHP microbial lethality when the a_w values of foods are increased (Morales, Calzada, & Nuñez, 2006). For instance, increasing the a_w of dry-cured ham from 0.860 to 0.960 led to more than 4 log-reductions of *L. monocytogenes* cells (Bover-Cid et al., 2015), shown in Figure 2.1b. Hayman, Kouassi, Anantheswaran, Floros,

and Knabel (2008) investigated the effect of a_w on *L. monocytogenes* inactivation by comparing pressure treatments at 600 MPa for 5 min in lyophilized cells and in cells suspended in different peptone water/glycerol solutions. No survivors were detected in solutions with a_w between 0.99-0.86 (6.5-7.5 log reductions), there were only 2.5 log reductions when a_w = 0.83 and no significant reductions after pressure treatments of lyophilized cells.

The mechanisms of low a_w value protection on *L. monocytogenes* cells during pressure treatments are not clearly defined but have been associated with protein stabilization (Bover-Cid et al., 2015; Hayman et al., 2008). Together with technological parameters and food components, the food intrinsic factors pH and a_w play an important role in the efficacy of pressure treatments to inactivate *L. monocytogenes* cells.

2.5.3. Food preservatives

To cater for the current demand for more natural foods without compromising food quality and safety, the combination of HHP with the addition of natural preservatives has been considered (Alpas & Bozoglu, 2002). In this context, pressurization treatments may be applied to reduce/substitute the addition of chemical preservatives to foods, such as sodium chloride and nitrite (Valdramidis, Patterson, & Linton, 2015).

The combination of HHP with the addition of mint essential oil at 0.05 or 0.1 % v/v in a yogurt drink reduced the pressure treatment severity required by 100-300 MPa to achieve the same levels of *L. monocytogenes* inactivation as in individual applications of pressure treatments (Evrendilek & Balasubramaniam, 2011). The addition of mint essential oil led to an additional increase in inactivation of more than 1 log cfu/mL, resulting in more than 6 log-reductions of *L. monocytogenes* at 600 MPa for 5 min. HHP and essential oils have similar effects on microbial structures, i.e. membrane damage (Gayán et al., 2012), and their combination should be considered as an alternative to the combination of mild heat with HHP processing in products which are sensitive to heat and elevated pressures.

Long pressure-holding times were required to achieve reductions in *L. monocytogenes* in deionized water corresponding to those achieved in nisin solutions during pressure treatments,

indicating that cell pressure-inactivation can be accelerated by adding nisin to foods (Gou, Lee, & Ahn, 2010). Accordingly, Hereu, Bover-Cid, Garriga, and Aymerich (2012) concluded that applying nisin to sliced dry-cured ham in combination with HHP was effective in inactivating *L. monocytogenes*.

Balamurugan et al. (2016) demonstrated that high pressure-induced inactivation of *L. monocytogenes* in ground chicken can be influenced by the type of salt and its concentration. Increasing NaCl from 0 to 2.5 % w/w at 600 MPa for 1 min led to lower reduction values (6.16 to 1.29 log cfu/g), which could be associated with the effect of decreasing a_w values, discussed previously. At the same pressure level, an increase in $CaCl_2$ concentration from 0 to 2.5 % w/w resulted in an increase of more than 1 log-unit reduction on *L. monocytogenes* populations.

The combination of potassium sorbate at 0.1 % w/w with HHP at 350 MPa for 20 min led to approximately 6 log reductions of *L. monocytogenes* cells in Indian white prawn muscle, while the application of potassium sorbate individually resulted in just 1 log reduction of cells (Das et al., 2016). Controversially, the addition of potassium lactate at 1.8 % w/w during HHP processing did not significantly enhance the inactivation of *L. monocytogenes* in ready-to-eat poultry meat (Lerasle et al., 2014).

These studies demonstrate the importance of considering the effect of food composition, in combination with different chemical and natural preservatives, on reducing *L. monocytogenes* levels during HHP treatments as the additives could interact with the food matrix and affect the final levels of inactivation.

2.6. Modelling the effect of *L. monocytogenes* culture physiology

Knowledge of the history of a bacterial culture is essential when setting HHP conditions as the growth stage of the cells, their physiological state, concentration, growth temperature and strain variability have been shown to affect HHP- induced inactivation (Hayman, Anantheswaran, & Knabel, 2007; Juck et al., 2012; McClements, Patterson, & Linton, 2001; Muñoz-Cuevas et al., 2013).

Saucedo-Reyes et al. (2009) studied the lethal effect of HHP on *L. innocua* at both exponential and stationary phases in TSB reference medium. Models analysed indicated that cells in the stationary phase showed significantly lower inactivation rates (~ 6 log-unit reductions at 400 MPa/15 min) and were more resistant to pressures compared with cells in the exponential phase (~ 7 log-unit reductions at 400 MPa/15 min). This is in accordance with studies conducted on milk (McClements et al., 2001) and tryptose broth (Tay et al., 2003) but is at variance with the study on turkey meat by Juck et al. (2012), in which exponential phase cells were more resistant than stationary phase cells during HHP treatments at 400 and 600 MPa for 2 min at different temperatures, reaching lower log-reductions during treatments.

A possible explanation for these differences is that cells grown on liquid foods or broth are mostly found in planktonic form. With the aging of the culture, they would therefore respond to starvation stress by producing proteins that increase their pressure-resistance at the stationary phase (Juck et al., 2012). On the other hand, in the study by Juck et al. (2012) cells were grown to exponential or stationary phases in turkey meat, i.e. a solid food matrix, and, according to the authors, may not have been evenly distributed over its surface, so that the progressive decrease in oxygen and nutrient levels would result in more sensitive cells at the late stationary phase.

Another hypothesis put forward to explain differences in the pressure resistance of cells grown in solid and liquid media and at different phases of growth is that in solid food matrices cell growth may be confined to the food structure, reaching lower maximum population densities and consequently becoming more sensitive to pressures at the late stationary phase.

Regarding the effect of the initial level of contamination on HHP efficacy, it has been documented that an increase in cell concentration leads to a decrease in log reductions achieved with pressure treatments. Youart et al. (2010) developed a mathematical model (Equation 2.6) to predict the time taken to inactivate (TTI) *L. monocytogenes* in TSB based on pressure (450 to 700 MPa), the inoculum level (2 to 6 log cfu/L), sodium chloride (1 or 2 %), and sodium lactate (0 or 2.5 %) at 4 °C. TTI increased with a rising inoculum level and decreasing pressure magnitude, which were the only significant factors among those evaluated.

$$\ln TTI = 1.3806 + 0.6942 \cdot IC - 0.2416 \cdot P + 0.1942 \cdot IC \cdot P + 0.2934 \cdot P^2 \quad \text{Eq. (2.6)}$$

where *TTI* is time to inactivation; *IC* is the initial level of contamination; and *P* is the pressure level.

The pressure susceptibility of *L. monocytogenes* varies between different strains (Alpas et al., 1999). Tay et al (2003) compared the sensitivity of *L. innocua* and 9 strains of *L. monocytogenes* in tryptose broth. Among the strains, *L. innocua* showed intermediate resistance, indicating that the strain evaluated should not be used as a *L. monocytogenes* surrogate in that condition since other strains showed higher barotolerance. *L. monocytogenes* OSY-8578 and Scott A were the most resistant and labile to pressures, respectively, and their death inactivation kinetics were evaluated at pressures varying from 350 to 800 MPa during 1-20 min at 30 °C. In both cases, similar non-linear tailing inactivation behaviour was obtained, with ~ 7 log reductions of *L. monocytogenes* OSY-8578 and > 8 log reductions of Scott A at 350 MPa for 20 min.

In the food industry, *L. monocytogenes* cells are likely to be stressed due to harsh conditions, i.e. low environmental temperatures (Guillier & Augustin, 2006). It is important to address the influence of the physiological state of cells on *L. monocytogenes* baroresistance. In the modelling approach of Hereu, Dalgaard, Garriga, Aymerich, and Bover-Cid (2014), freeze-stressed cells were more resistant to pressures than cold-adapted cells during pressurization treatments at 400 MPa (5 min/15 °C). *L. monocytogenes* cold-stressed cells grown at 8 °C were more pressure-resistant than cells grown at 20 or 32 °C on TSB treated at 500 MPa for different exposure times (Teixeira et al., 2016). Furthermore, studies have shown that *L. monocytogenes* cells submitted to HHP processing may exhibit resistance to different stresses and varying degrees of resistance among a population as they are genetically flexible (Van Boeijen, Francke, Moezelaar, Abee, & Zwietering, 2011).

Differences in pressure sensitivities due to differences in culture conditions and between cells of the same population indicate the need for stochastic modelling studies that take into account the variability among *L. monocytogenes* cells representative of food industry scenarios. This

variability may influence cell recovery during the storage of pressure-treated foods and, consequently, on food safety.

2.7. Future Challenges

In most of modelling approaches, isobaric and isothermal conditions are assumed during HHP application. In such cases, the pressure increase during come-up times (i.e. time to achieve the pressure levels set in the experimental design) and the temperature rise due to adiabatic heating are not taken into account. However, authors have reported significant *L. monocytogenes* reductions in foods during come-up times (Lucore, Shellhammer, & Yousef, 2000) and its impact must be incorporated in the design of HHP, so the complete microbial kinetics can be described (Valdramidis et al., 2007).

Few modelling approaches have considered the dynamic character of HHP technology (Koseki & Yamamoto, 2007b; Valdramidis et al., 2007). Therefore, further research taking into account the system specificity and variability for process design and optimization is needed, to obtain more realistic and reliable HHP inactivation models, which cannot be accomplished by conventional curve-fitting procedure.

2.8. Conclusions

Inactivation kinetics of *L. monocytogenes* in foods as a function of pressure-holding times has been described by primary linear and non-linear models. Among the non-linear equations, the Weibull, log-logistic, Baranyi and Gompertz are the most commonly used to fit the inactivation curves. Polynomial equations generated based on Response Surface Methodology are usually applied to study the influence of technological parameters, food components, intrinsic factors and food additives in pressure-induced inactivation of *L. monocytogenes*. This review discusses the most important factors that influence *L. monocytogenes* inactivation by high hydrostatic pressure. However, some studies are controversial on whether one factor (i.e. fat content) exerts a protective effect on bacteria or acts synergistically to increase the lethality of pressure

treatments. The development of more realistic modelling approaches considering the dynamic character of HHP, accounting with the impact of pressure come-up times and adiabatic heating during process is encouraged. Finally, although high hydrostatic pressure is effective for reducing *L. monocytogenes* levels in foods, the recovery of injured cells during storage of pressure-treated products must be taken into consideration in order to evaluate the real impact of this novel technology on food safety.

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Chapter III

Chapter III: Mathematical approach for the *Listeria monocytogenes* inactivation during high hydrostatic pressure processing of a simulated meat medium

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3.1. Abstract

Based on a central composite experimental design, the effects of five factors: pH (4.6-6.6), sodium chloride (NaCl, 0–10 %), sodium nitrite ($NaNO_2$, 0–152 ppm), pressure (P, 300–600 MPa) and pressure-holding time (t, 0–10 min) on the inactivation of *Listeria monocytogenes* by high hydrostatic pressure processing on a simulated meat medium (SMM) were evaluated and quantified. Results showed that *L. monocytogenes* reductions during pressure treatments ranged from 0 to more than 6 log cfu/g. A polynomial inactivation model was developed being P, t and NaCl the only significant factors influencing HP-lethality ($p \leq 0.05$). Inactivation on SMM increased with the increase in P and t, and a synergistic effect between both factors on process effectiveness was remarked. By increasing NaCl concentration (*i.e.* lowering a_w values), a baroprotective effect on *L. monocytogene*s cells was evidenced. Besides the well-known effects of technological parameters, this study highlights the relevant influence of meat products formulations on the inactivation of *L. monocytogenes* induced by HHP processing.

Keywords: microbial inactivation, predictive microbiology, modelling, meat products, high pressure, food safety

3.1. Introduction

The occurrence of *Listeria monocytogenes* in food production facilities, especially in the meat industry, requires constant control and focus by food operators and risk managers (Buchanan, Gorris, Hayman, Jackson, & Whiting, 2017). Although improved control measures have greatly reduced the prevalence of *L. monocytogenes* in foods, the rate of illness has not decreased during the last decade (Buchanan et al., 2017). In the European Union (EU) there was an increasing trend of listeriosis from 2008 to 2015, with cases reported in 28 member states in 2015 (EFSA, 2016). At the same year the fatality rate was 17.7 % among the 1,524 listeriosis confirmed cases with known outcome (EFSA, 2016). Meat products with relatively long shelf-life, such as cooked sausages, cooked sliced ham and fermented salami, are mainly included among the foods implicated in listeriosis cases worldwide (Nastasijevic et al., 2017).

In accordance with the European Regulation (EC) No 2073/2005, manufacturers should demonstrate that *L. monocytogenes* levels on ready-to-eat (RTE) meats which are unable to support its growth should not exceed 100 cfu/g by the end of their shelf-life (European Commission, 2005). In order to comply with microbiological criteria, high hydrostatic pressure (HHP) processing technology has been proposed as a nonthermal method to inactivate *L. monocytogenes* in RTE meats, without compromising their organoleptic properties and nutritional value (Bover-Cid, Belletti, Aymerich, & Garriga, 2015; Bover-Cid, Belletti, Garriga, & Aymerich, 2011; Hereu, Dalgaard, Garriga, Aymerich, & Bover-Cid, 2012; Rubio, Possas, Rincón, García-Gímeno, & Martínez, 2018). Some HHP-treated meat products such as pork and poultry cuts, whole and sliced ham, chicken and turkey products, and chorizo sausage are currently available at retail points in Spain (Sun & Holley, 2010).

The development of predictive mathematical models describing microbial inactivation on RTE meats during pressure treatments represents a positive asset for food safety as they provide science-based methods supporting the validation of HHP treatments at the industrial level (Bover-Cid, Belletti, Aymerich, & Garriga, 2017). Several models expressing the reductions in

L. monocytogenes levels in foods during pressure treatments are available in literature (Possas, Pérez-Rodríguez, Valero, & García-Gimeno, 2017). Research has highlighted that the simultaneous influence of many factors on HHP lethal effectiveness (*i.e.* processing parameters, food characteristics, physiological state of bacterial cells) arises the need of product-oriented approaches in order to develop more realistic and precise predictive models of microbial inactivation (Bover-Cid et al., 2017; Hereu, Dalgaard, et al., 2012; Rubio et al., 2018). Nonetheless, the development of product-oriented approaches might be very time-consuming and expensive considering the wide variety of meat and meat products available. Alternatively, general modelling approaches in which laboratory media could simulate the characteristics of meat products have been developed, although their validation in the target food before application is essential (Possas et al., 2017).

Predictive inactivation models have been developed for pressure treatments in liquid laboratory media that can mimic the microbial environment of liquid foods or water-oil emulsions, such as tryptone soya broth (Muñoz-Cuevas et al., 2013; Saucedo-Reyes, Marco-Celdrán, Pina-Pérez, Rodrigo, & Martínez-López, 2009), brain heart infusion broth (Dogan & Erkmen, 2004; Valdramidis, Patterson, & Linton, 2015) and peptone solution (Buzrul & Alpas, 2004; Buzrul, Çevik, & Alpas, 2008). In these cases, the environment is relatively uniform in terms of nutrients and metabolites while microorganisms are typically encountered in planktonic form (Koutsoumanis, Kendall, & Sofos, 2004). However, in solid foods microorganisms may also be encountered on product surfaces or distributed inside food microstructure (Koutsoumanis et al., 2004), which would limit the application of models generated in liquid media. Furthermore, the a_w of liquid culture media, a very important intrinsic factor influencing microbial inactivation by HHP, would not be realistic for solid foods, such as RTE meats. To date, there is no HHP predictive model developed in a solid laboratory medium that would simulate the composition and physicochemical properties of actual RTE meats.

In the present study the influence of pH, sodium chloride, sodium nitrite, pressure and pressure-holding time on *L. monocytogenes* inactivation by HHP was studied and modelled on a

simulated meat medium. The inactivation model generated covers a wide range of the technological parameters and the meat inherent factors evaluated.

3.2. Material and Methods

3.2.1. Experimental design

The effect of five factors on *L. monocytogenes* inactivation on simulated meat medium was studied on the basis of a central composite experimental design (CCD). The factors evaluated were sodium chloride (NaCl), pH, sodium nitrite ($NaNO_2$), pressure (P) and pressure-holding time (t). The experimental layout is shown in Table 3.1. A total of 44 experiments were randomly performed in order to exclude the disturbing effects of environmental conditions (Joglekar & May, 1987; Robinson, 2000). Additional experiments were conducted at the central point of the CCD to enable the evaluation of the experimental error and the lack-of-fit of the model.

The levels of factors defined for this study are in line with current legislations, meat producer's information and literature. The experimental range for sodium nitrite was 0–152 ppm. The minimum level, set to 0, was established based on the current demand/production for RTE meats without its addition, while the maximum was in line with the legislation for low salt content foods, which establishes a maximum of 150 mg/kg (ppm) of meat for low salt content products (European Commission, 2006). The pH range (4.6–6.6) is representative of a variety of RTE meats including fermented ones (Casquete et al., 2011; Fonseca, Cachaldora, Gomez, Franco, & Carballo, 2013; Hwang et al., 2009). The range of NaCl (0–10 %) was defined based on the possibility of its absence on meat products and on its maximum concentration on dry-matter of cured meats (FSIS, 2011). Finally, studies have shown that pressure treatments between 300 and 600 MPa for up to 10 min are able to inactivate bacteria in meat products and their application is commercially feasible (Bover-Cid et al., 2017; Possas et al., 2017).

Table 3.1- Central composite experimental design for studying the effect of the selected factors on the inactivation of *Listeria monocytogenes* on simulated meat medium

Factors	**Symbols**		**Levels**				
	Uncoded	**Coded**	**-α**	**-1**	**0**	**+1**	**+α**
pH	pH	X_1	4.6	5.2	5.6	6.0	6.6
NaCl (% w/w)	NaCl	X_2	0	2.9	5.0	7.1	10.0
$NaNO_2$ (ppm)	$NaNO_2$	X_3	0	44.0	76.0	108.0	152.0
Pressure (MPa)	P	X_4	300.0	387.0	450.0	513.0	600.0
Time (min)	t	X_5	0	3.0	5.0	7.0	10.0

3.2.2. Bacterial strains and culture preparation

A four-strain cocktail of *L. monocytogenes* was prepared for samples inoculation. Three strains of *L. monocytogenes* were isolated from dry-fermented meat products and the fourth one was obtained from the Spanish Type Culture Collection (CECT 935, serovar 4b). The CECT 935 is a reference strain as per recommended in UNE-CEN ISO/TS 11133 and EN ISO 11290.

Stock cultures were maintained by regular subculture on Plate Count Agar (PCA, Oxoid, UK) and stored at 4 °C. Before each experiment, a loopful of each stock culture was transferred to Brain Heart Infusion broth (BHIB, Oxoid, UK) and incubated at 37 ºC, for 18 h, yielding early stationary phase cultures. The cocktail was prepared by mixing equal volumes of the four individual cultures in peptone water (0.1 % w/v) (Oxoid, UK). Finally, the cocktail was serially diluted in saline solution in order to reach a final concentration of 10^6-10^7 cfu/g in the modified agars.

3.2.3. Samples preparation

Brain Heart Infusion agar (BHIA) (Oxoid, UK) was modified to simulate meat products by adding 18 g/L of glucose and 3 g/L of yeast extract (Oxoid code LP12, UK) (Devlieghere, Debevere, & Van Impe, 1998a). In previous studies, BHIB with these modifications was considered suitable to simulate cooked and cured meat products characteristics (Devlieghere et al., 1998a; Devlieghere, Debevere, & Van Impe, 1998b; Devlieghere, Lefevere, Magnin, & Debevere, 2000; Valdramidis et al., 2015). Glycerol (PanReac, Spain) was also added at a concentration of 5 % (v/v) to reduce the a_w of samples to a range of values more representative of cured-fermented meat products. The glycerol concentration was uniform for all treatments and it was adjusted based on previous tests confirming that it does not influence on *L. monocytogenes* growth and survival on BHIA (data not shown). Before medium sterilization, NaCl (Merck Millipore, Germany) was added to reach the concentrations set in the experimental design (Table 3.1). The pH of the modified BHIA was adjusted with HCl (1N) and monitored before and after sterilization and also after HHP treatments by using the pHmeter Edge HI2020

(Hanna Instruments, USA). After sterilization in autoclave (121 °C/15 min), filter-sterilized concentrated solutions of $NaNO_2$ (Merck Millipore, Germany) were added to modified BHIA to attain the target values. Media were then inoculated by aseptically adding aliquots of the *L. monocytogenes* cocktail. Subsequently, 25-mL samples were transferred to sterile polyethylene casings and Fisherbrand Blender bag clips (Thermo Fisher, Spain) were placed in the casings, avoiding air entrance until agar solidification at room temperature. After solidification, the bag clips were removed and the casings were then heat-sealed with the aid of a sealer (TEW 5800041, Spain). The a_w of samples was monitored using a Decagon CX-2 Aqualab hygrometer (Decagon Devices Inc., USA) at 20 ºC.

3.2.4. High hydrostatic pressure processing

Simulated meat samples were pressurized at the target pressure intensities and pressure holding-times corresponding to the CCD trials in an industrial hydrostatic pressure unit (Wave 6000/135, NC Hyperbaric, Burgos, Spain), equipped with a 135-L high-pressure vessel. The pressure transmitting fluid was additive-free water with an initial temperature of 18 °C. The treatment pressures were reached in approximately 4 min and decompression was instantaneous.

3.2.5. Microbiological analysis

L. monocytogenes counts were obtained prior to and immediately after HHP treatments on simulated meat samples. For microbial determinations, sample casings were aseptically opened with the aid of a sterile lancet and the whole samples were transferred to sterile stomacher bags. The stomacher bags were filled with buffered peptone water (1:10) (Oxoid, UK) and homogenised in Stomacher (IUL, Spain) for 1.5 min. The homogenate was serially diluted in saline solution (0.85 % w/v) and plated onto the selective media PALCAM (Oxoid, UK) and incubated at 37 ºC ± 1°C for 48 h ± 3 hours. In order to decrease the detection limit in high-pressured samples, 5-mL aliquots were pour plated in macro PALCAM dishes (140 x 20 mm).

For expected counts below the limit of quantification (< 2 cfu/g), the presence or absence of *L. monocytogenes* was determined by enrichment following standard methods (ISO, 2004). For modelling purposes, positive results below the quantification limit were recorded as 0 log cfu/g (< 1 cfu/g), while absence in 25 g was computed as -1.40 log cfu/g (< 1 cfu/25g). The concentration of *L. monocytogenes* in samples was determined at least in duplicate for each combination of the CCD. Bacterial inactivation was evaluated in terms of logarithmic reductions as the difference between counts of HHP-treated samples (*N*, log cfu/g) and counts of the control samples, which were not submitted to HHP treatments (N_0, log cfu/g)- (*i.e.* log N/N_0).

3.2.6. Mathematical modelling

The response surface methodology (RSM) was the empirical procedure followed to study the relationship between the selected independent variables (NaCl, $NaNO_2$, pH, P and t) and the dependent variable (*i.e.* inactivation of *L. monocytogene*s on simulated meat, log N/N_0). The statistical package Statistica® for Windows (version 11, Statsoft Inc., USA) was used for mathematical modelling. Prior to model development, the values of the independent variables were normalized by coding according to Table 2.1. To generate the equation that best fitted to the experimental data without compromising parsimony, the backward stepwise regression method was conducted and only the statistically significant ($p \leq 0.05$) terms deriving from each factor were maintained in the final model. The goodness-of-fit and the statistical significance of the model were evaluated by means of the adjusted determination coefficients (R^2_{adj}) and the significance *p–values* derived from the *F*–test. Response surface graphs were drawn in which the independent variable (studied factor) not shown remained at the central point of the CCD.

3.2.7. Model performance assessment

The accuracy factor (A_f) and bias factor (B_f), considered as measures of the performance of predictive models, were calculated to evaluate the capability of prediction of the model (Baranyi, Pin, & Ross, 1999; Ross, 1996). The A_f indicates the spread of observed data in

comparison with the predictions of the model, while the B_f is a measure of the extent to which the model under- or overestimates the inactivation observed.

3.3. Results and Discussion

3.3.1. Inactivation of *L. monocytogenes* by HHP on simulated meat medium

The inactivation data expressed as logarithmic reductions (log N/N_0) of viable cells achieved for each of the 44 experiments conducted in accordance with the CCD are summarized in Table 3.2. In general, *L. monocytogenes* inactivation ranged between 0.00 (Trial 11) and -6.20 log cfu/g (Trial 9). In the present study, *L. monocytogenes* could be detected in all trials, excepting in trial 18 in which samples had the lowest pH value evaluated (4.6).

The experimental results revealed that the pressure resistance of *L. monocytogenes* on simulated meat medium was significantly lower than that usually reported in RTE meats. A reduction of approximately 4 log cfu-g was achieved with the application of high-pressure treatments at 600 MPa for 5 min on Genoa salami, with a_w= 0.94, contaminated with a 5-strain cocktail of *L. monocytogenes* at a concentration of 6 log cfu/g (Porto-Fett et al., 2010). At the same pressure-holding conditions, a reduction of more than 6 log cfu/g was achieved in the studied simulated meat medium, with the same a_w (NaCl = 5 % m/v) (Trial 9, Table 3.2). Furthermore, the application of 450 MPa for 5 min led to nearly 2.5 and 1.0 log cfu/g reductions of *L. monocytogenes* in cooked ham and mortadella, respectively (both with a_w = 0.98) (Hereu, Dalgaard, et al., 2012), while at the same conditions, a reduction of 6.12 log cfu/g was attained in the simulated meat medium under study (Trial 16, Table 3.2). These differences in inactivation levels may be attributed to differences in the composition of the products evaluated. For instance, fat exert a protective effect on bacteria cells during pressurization of foods, by means of mechanisms that are not well known (Ferreira, Almeida, Delgadillo, Saraiva, & Cunha, 2016; Possas et al., 2017). The simulated meat medium has lower fat content in comparison with actual meat products, which might have led to a lower pressure-resistance of *L. monocytogenes* cells in our study.

Table 3.2- *Listeria monocytogenes* inactivation (log N/N_0) results obtained on simulated meat medium, for each trial of the central composite design

Trial	pH	NaCl (%)	$NaNO_2$ (ppm)	Time (min)	Pressure (MPa)	Inactivation[a] (log N/N_0)
1	5.2	7.1	44	3	513	-4.51 (0.09)
2	6.0	2.9	108	3	513	-5.10 (0.02)
3	5.2	7.1	108	3	513	-4.69 (0.02)
4	6.0	7.1	108	3	513	-4.72 (0.22)
5	5.2	2.9	44	3	513	-4.96 (0.03)
6	6.0	7.1	44	3	513	-4.41 (0.23)
7	6.0	2.9	44	3	513	-5.10 (0.03)
8	5.2	2.9	108	3	513	-4.95 (0.02)
9	5.6	5	76	5	600	-6.20 (0.10)
10	5.6	5	76	10	450	-5.74 (0.06)
11	5.6	5	76	0	450	0.00 (0.00)
12	5.6	5	76	5	300	-3.52 (0.04)
13	6.6	5	76	5	450	-5.01 (0.00)
14	5.6	5	76	5	450	-5.05 (0.02)
15	5.6	5	152	5	450	-5.54 (0.10)
16	5.6	0	76	5	450	-6.12 (0.00)
17	5.6	5	0	5	450	-5.02 (0.06)
18	4.6	5	76	5	450	-5.31 (0.09)
19	5.6	10	76	5	450	-3.80 (0.02)
20	5.6	5	76	5	450	-5.09 (0.02)
21	6.0	2.9	108	3	387	-4.32 (0.09)
22	6.0	7.1	44	3	387	-3.44 (0.07)
23	6.0	7.1	108	3	387	-3.33 (0.13)
24	5.2	7.1	108	3	387	-3.12 (0.26)
25	5.2	2.9	44	3	387	-4.57 (0.19)
26	6.0	2.9	44	3	387	-4.32 (0.00)
27	5.2	7.1	44	3	387	-3.01 (0.14)
28	5.2	2.9	108	3	387	-4.04 (0.09)
29	6.0	2.9	108	7	387	-4.76 (0.02)
30	5.2	2.9	44	7	387	-4.95 (0.00)
31	5.2	7.1	44	7	387	-3.44 (0.05)
32	5.2	7.1	108	7	387	-3.67 (0.61)
33	6.0	7.1	108	7	387	-3.49 (0.01)
34	6.0	7.1	44	7	387	-3.81 (0.00)
35	6.0	2.9	44	7	387	-4.76 (0.14)
36	5.2	2.9	108	7	387	-4.54 (0.04)
37	5.2	7.1	108	7	513	-5.45 (0.13)
38	6.0	2.9	44	7	513	-5.99 (0.02)
39	5.2	2.9	108	7	513	-5.51 (0.03)
40	5.2	2.9	44	7	513	-5.44 (0.19)
41	5.2	7.1	44	7	513	-5.49 (0.35)
42	6.0	2.9	108	7	513	-5.99 (0.00)
43	6.0	7.1	44	7	513	-5.35 (0.12)
44	6.0	7.1	108	7	513	-5.22 (0.00)

[a] Mean of replicates/Standard deviation reported in parenthesis.

Besides composition, other factors that might influence the *L. monocytogenes* pressure-resistance on meat products are the physiological state of cells and the initial level of contamination. Bover-Cid et al. (2011) selected the most resistant strain among ten *L. monocytogenes* strains previously tested for their pressure-resistance, to be used in their study of HHP inactivation. These authors reported reductions below 0.5 log cfu/g on dry-cured ham pressure-treated at 450 MPa for 5 min. The inoculum size also can influence on the inactivation rate by HHP processing as demonstrated in research published by different authors (Koseki, Mizuno, & Yamamoto, 2007; Koseki & Yamamoto, 2007; Youart, Huang, Stewart, Kalinowski, & Legan, 2010). Therefore, caution should be taken when comparing inactivation results derived from different approaches, due to differences in experimental design including target strains evaluated and their initial levels in meat products.

The lethality of the HHP process against *L. monocytogene*s increased with the increase in pressure and pressure-holding times. Relatively low *L. monocytogenes* inactivation was recorded at 300 MPa (-3.52 log cfu/g, Trial 12), while the highest inactivation level was achieved when 600 MPa was applied to simulated meat samples (-6.20 log cfu/g, Trial 9). In trials in which a pressure of 450 MPa was applied and sodium chloride concentration was intermediate (*i.e.* 5 %, a_w = 0.945), reductions were equal or higher than 5 log cfu/g. At the central points of the CCD (Trials 14 and 20), 5 min treatments also resulted in nearly 5-log unit reductions, though the increase of treatments duration from 5 to 10 min (Trial 10) did not lead to significant increase on inactivation levels. HHP processing application in this study is in accordance with FDA recommendations for non-pasteurization methods, which establishes a reduction of at least 5-log units of the target microorganism in foods (Saucedo-Reyes et al., 2009). Moreover, HHP application is in accordance with the guidelines of the Food Safety Inspection Service/United Sates Department of Agriculture for controlling *L. monocytogenes* in post-lethally exposed RTE meat products, which signals that manufacturers applying this technology for reprocessing contaminated products should ensure that at least 5-log reductions are achieved (FSIS, 2014). The safety criteria by the Spanish Agency of Food Safety for RTE

meat products, set at 4-log reductions of *L. monocytogenes*, was also achieved in this study (AESAN, 2005).

The effects of $NaNO_2$ on *L. monocytogenes* pressure-induced inactivation were not apparent in the present study. In Trials 15 and 17, the increase in sodium nitrite concentration from 0 to 152 ppm while maintaining the values of the other four factors fixed at the central point of the CCD, led to the increase of 0.5 log cfu/g on *L. monocytogenes* reductions, value lower than the threshold considered relevant (*i.e.* 1 log) (Bover-Cid et al., 2017; FSIS, 2014). In meat products, the increased reactivity of $NaNO_2$ with meat proteins results in low residual levels during relatively short storage period. Indeed, it has been reported that only 10-20 % of the total added $NaNO_2$ can remain in cured meat products (Alahakoon, Jayasena, Ramachandra, & Jo, 2015). The reactivity of the $NaNO_2$ on the simulated meat medium is assumed to be lower than in meat products, due to its lower protein content. Although this fact would contribute to the presence of a higher concentration of residual $NaNO_2$ on the medium studied, nitrite was not identified as a significant factor influencing on *L. monocytogenes* inactivation levels, thus highlighting that the induced pressure conditions produced much higher inhibition on the target microorganism than its addition alone. In accordance, the inhibitory effect of $NaNO_2$ was neglected in comparison with the induced pressure holding conditions on cooked pork ham (Pietrzak, Fonberg-Broczek, Mucka, & Windyga, 2007).

There is scarce information on the combined effects of nitrite with physical stress treatments on bacterial vegetative cells (De Alba, Bravo, Medina, Park, & Mackey, 2013). There is evidence that pressure application may lead to the development of reactive oxygen species (ROS) within cells (Aertsen, De Spiegeleer, Vanoirbeek, Lavilla, & Michiels, 2005). When encountered in its radical form (NO^-), nitrite can react with ROS, resulting in the formation of enhanced antimicrobial molecular species. Thus, it was expected that HHP processing application would increase the antimicrobial effect of nitrite (De Alba et al., 2013). Besides, the bactericidal effect of nitrite seems to increase during storage of pressure-treated foods. This synergistic action was not evidenced in the present study, but it was clear in the studies of Valdramidis et al. (2015)

and De Alba et al. (2013). Divergences between studies may be attributed to the characteristics of the simulated meat evaluated, as the antimicrobial effect of nitrite depends, among other factors, on pH and salt concentration (De Alba et al., 2013).

Regarding pH, no statistical differences were detected for control and HHP-treated simulated meat samples ($p > 0.05$). Similarly to what was observed on trials with extreme sodium nitrite concentrations, the difference on reductions on the trials with the highest and lowest pH values (13 and 18) was just 0.30 log cfu/g on average. Although in this work the decrease in pH from 6.6 to 4.6 did not lead to significant reductions of *L. monocytogenes* levels during pressurization, it is expected that the increase in the acidity of foods results in higher inactivation during HHP processing (Ferreira et al., 2016). For instance, at acid pH, nitrite is encountered in this radical form, exhibiting higher inhibitory effects on bacteria, which associated with high-pressures would exert additional bactericidal action in foods such as fermented cured meats in which its addition is allowed (De Alba et al., 2013). Furthermore, the recovery capacity of pressure-injured cells during storage of pressure-treated acidic foods is reduced (Ferreira et al., 2016).

A plausible explanation for the observed slight effect of pH levels on *L. monocytogenes* inactivation could be the combined effect of NaCl and pressure conditions, which could disguise the potential inhibition due to acidic pH of the simulated meat medium. In the study of Cheftel and Culioli (1997), the synergistic effect between NaCl and pressure produced significant inhibitory activity on *L. monocytogenes*, while other authors found out that HHP treatments at 600 MPa for 5 min had no antimicrobial effect against *L. monocytogenes* in sliced fermented sausages with no added sodium salt (Marcos, Aymerich, Garriga, & Arnau, 2013). Other factors such as chemical composition and physical structure of the agar medium could also have an influence on the low effect of pH on *L. monocytogenes* inactivation. According to the results obtained, these effects may be further studied in solid media.

The increase in NaCl concentration on simulated meat samples led to a linear decrease on a_w values (Figure 3.1). An ANOVA revealed that NaCl concentration was the only factor influencing on a_w values of samples ($p \leq 0.05$), which increased from 0.91 to 0.98 when sodium chloride concentration decreased from 10 to 0 %. At this a_w range, inactivation levels increased from -3.80 to -6.12 log cfu/g, while maintaining the other factors at the central point value (Trials 19 and 16). The a_w range of the simulated meat medium was set to represent for meat products such as *semi-dry sausages*, which exhibit a_w values from 0.95–0.97, and *dry sausages*, with values of 0.91–0.93 (FSIS, 2011).

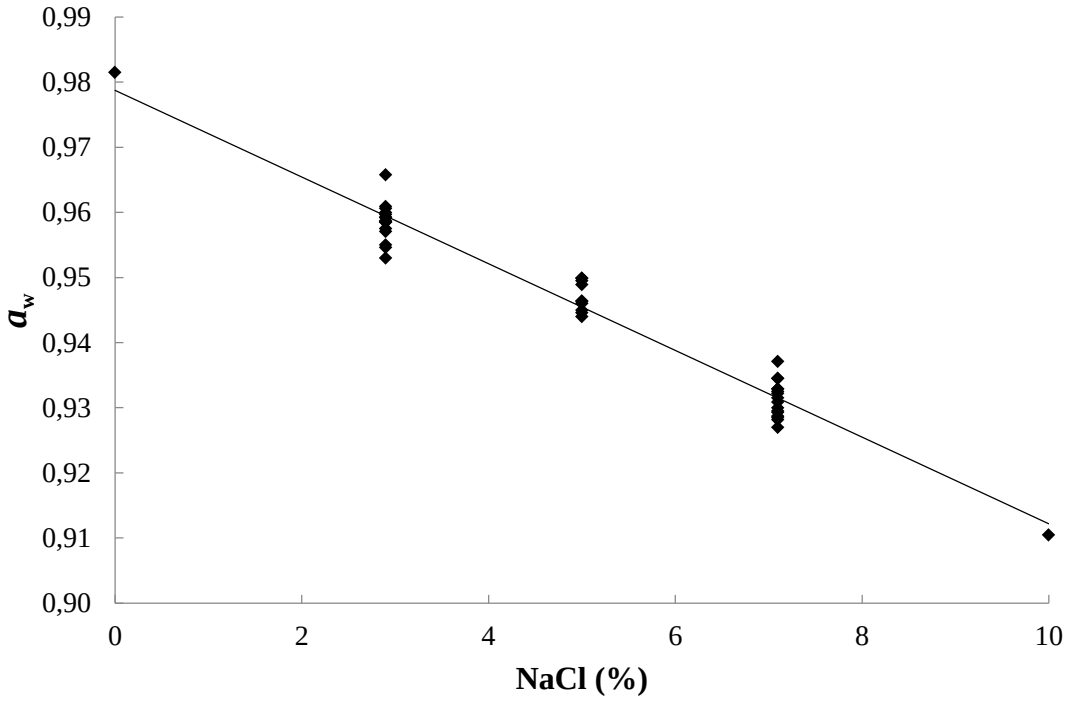

Figure 3.1- Relationship between a_w values and NaCl concentration in simulated meat medium samples with glycerol added at 5 % (R^2= 0.96)

Research by other authors have revealed that there is a marked increase on the lethality of HHP when the a_w of foods is increased (Bover-Cid et al., 2015, 2017; Hereu, Bover-Cid, Garriga, & Aymerich, 2012; Rubio et al., 2018). Rubio et al. (2018) found out that an increase in a_w values of Spanish chorizo sausage from 0.79 to 0.92 led to a reduction of approximately 2 log cfu/g of

L. monocytogenes levels during HHP application. Furthermore, an increase of the a_w of dry cured ham from 0.86 to 0.96 resulted in an increase on *L. monocytogenes* inactivation levels from -2.24 to -6.82 log cfu/g (Bover-Cid et al., 2015). These findings indicate that HHP processing can work as an effective preservation method, enabling a reduction in salt amounts added in RTE meat products formulation, as pressurization lethality is enhanced at higher a_w.

The mechanisms of the baroprotective effect of *L. monocytogenes* cells at lower a_w are not clearly defined but have been associated with the stabilisation of macromolecules such as proteins as a result of the decrease of cell compressibility, caused by the increase of solutes concentration in the cytoplasm of bacterial cells (Bover-Cid et al., 2017; Georget et al., 2015; Possas et al., 2017).

3.3.2. Mathematical modelling

The application of RSM offers, based on parameter estimates, an empirical relationship between *L. monocytogenes* inactivation and the independent variables under study. The result of the modelling approach followed in this study is the quadratic polynomial model shown as Equation 3.1.

$$\log\left(N/N_0\right) = -4.30320 + 0.91526 \cdot NaCl - 4.19288 \cdot \log t + 3.95856 \cdot (\log t)^2 - 0.00326 \cdot P - 0.00158 \cdot NaCl \cdot P - 0.00657 \cdot P \cdot \log t \quad \text{Eq. (3.1)}$$

where $\log(N/N_0)$ is the logarithmic reduction of *L. monocytogenes*; NaCl is the concentration of sodium chloride (% w/w); P is the applied pressure (MPa); and t is the pressure-holding time (min).

The *F*–value = 128 indicates that the model is statistically significant ($p \leq 0.0001$), and an R^2_{adj} = 0.91 (R^2 = 0.92) reveals a satisfactory correlation between model predictions and the observed inactivation data. The lack of fit of the model was non-significant (*F*–value = 7.12, $p > 0.05$).

The backward stepwise regression revealed that among the five factors studied, three factors significantly influenced on *L. monocytogenes* inactivation during HHP: NaCl, pressure and

pressure-holding time. The significant factors are present in the model as linear and quadratic terms (Equation 1). Results of the regression analysis are shown in Table 3.3. Pressure intensities and sodium chloride concentration were the most important factors influencing *L. monocytogenes* inactivation by HHP on samples as indicated by effect estimates, although the pathogen was more sensitive to pressure than to NaCl changes.

Table 3.3- Results of the multivariate regression describing the effect of NaCl, pressure and pressure-holding times on *Listeria monocytogenes* inactivation due to HHP processing of simulated meat medium

Terms[a]	**Regression coefficients**	**Standard Error**	***t***	***p***
Intercept	-4.303	0.585	-7.356	0.000
NaCl	0.915	0.062	14.669	0.000
log t	-4.193	0.777	-5.395	0.000
(log t)^2	3.959	0.275	14.412	0.000
P	0.003	0.001	2.553	0.014
NaCl ·P	-0.002	0.000	-11.496	0.000
t ·P	-0.007	0.002	-4.202	0.000

[a] Only the statistically significant terms were kept in the model through the backward stepwise regression.

Despite the positive relationship between *L. monocytogenes* inactivation and treatments duration, when increasing pressure-holding times, a decrease of its effect on HHP-induced inactivation was verified, characterizing a non-linear relationship. To reflect adequately the effect of treatment time on pressure-induced inactivation, this variable was rescaled to logarithm values prior to modelling, as recommended by Koseki and Yamamoto (2007).

The interaction of the technological parameters pressure and NaCl was significant, whereby the increase of NaCl concentration (*i.e.* reduction of a_w) associated with an increase in pressure intensities resulted in the reduction of process lethality. At pressures higher than 590 MPa the increase in sodium chloride concentration from 0 to 10 %, at fixed pressure-holding times, resulted in the increase in inactivation, probably caused by the increase in cell osmotic stress associated with the high-pressure applied. The factors P and t also showed a significant interaction, indicating that an increase in both variables at fixed sodium chloride concentrations,

resulted in a synergistic effect on *L. monocytogenes* inactivation, which is in accordance with other developed models (Bover-Cid et al., 2011; Rubio et al., 2018).

The response surface graphs are shown in Figure 3.2. The surfaces were generated based on the Equation 1 and provide an overview of how the three significant factors studied influenced HHP-induced *L. monocytogenes* inactivation. The log reductions of *L. monocytogenes* followed a linear trend with the change of both sodium chloride and pressure. The baroprotective effect of the decrease on a_w is clearly seen in the linear decrease of the inactivation while sodium chloride concentration increases. The strong influence of the interaction between P and NaCl of the equation makes the surface plot to sharply decrease at higher pressure intensities and low salt concentrations (Figure 3.2a). The curvature of the surface in Figure 3.2b is attributed to the second order term of the independent variable pressure-holding time.

By fixing the value of NaCl concentration (*i.e.* 5 %) in Equation 1, it is possible to demonstrate that the inactivation kinetics of *L. monocytogenes* on samples would follow a non-linear trend, under constant pressure intensities (Figure3.2b). The trend observed on curves, characterized by a rapid decrease on number of cells in the first 5 min of treatments, followed by a slow down on inactivation (*i.e.* tailing phenomenon), may indicate the coexistence of subpopulations of *L. monocytogenes* with different pressure-resistances on simulated meat. An HHP-sensitive and an HHP-resistant fraction of *L. monocytogenes* cells were identified even when individual strain-cultures were submitted to pressure treatments (van Boeijen, Moezelaar, Abee, & Zwietering, 2008). However, the use of a bacterial cocktail in our study could also explain for the existence of different bacterial sensitivities to HHP treatments.

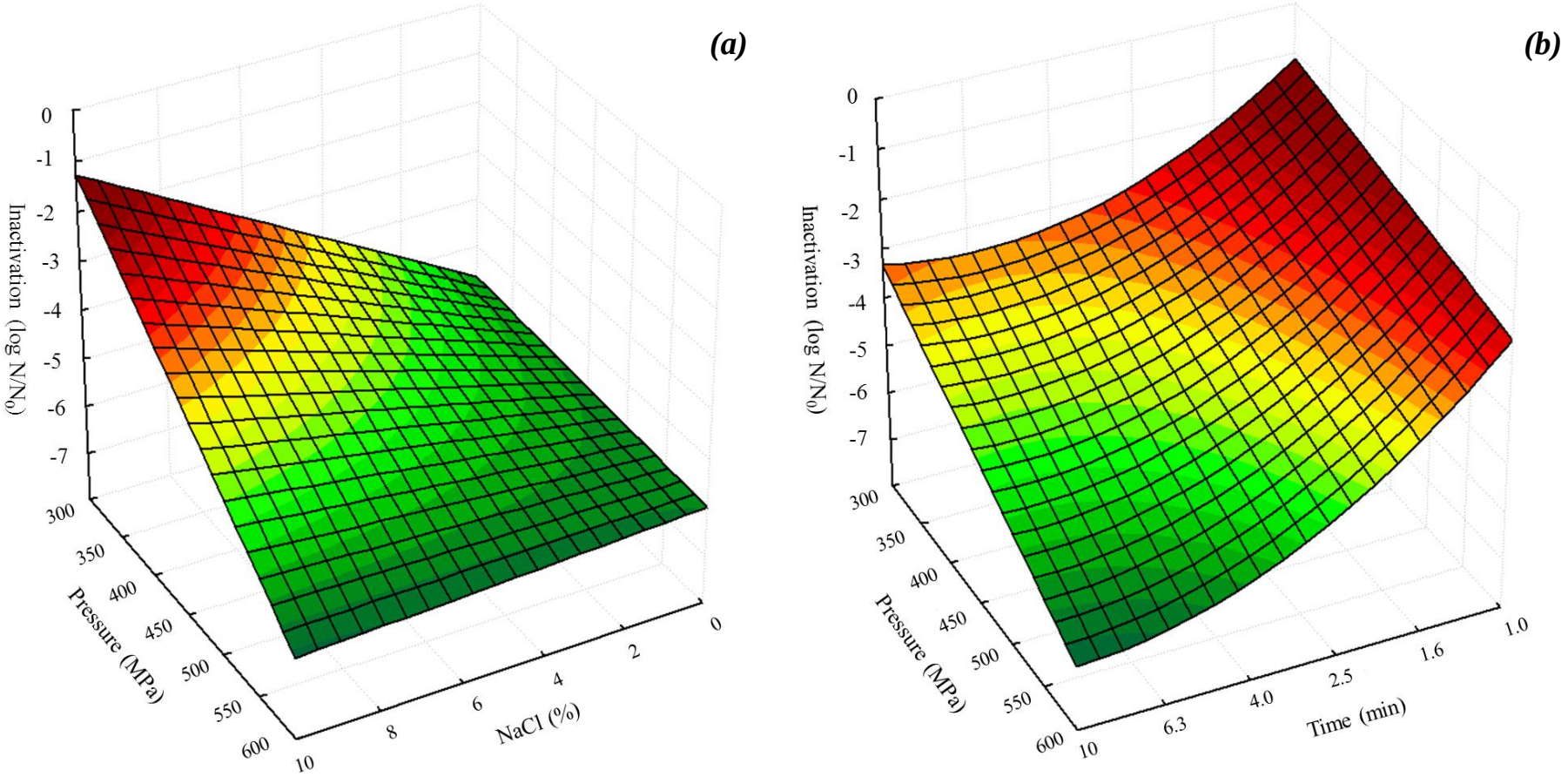

Figure 3.2- Response surface graphs of HHP-induced inactivation of *Listeria monocytogenes* in simulated meat medium samples, according to the developed model. (a) P and NaCl effects; (b) P and t effects. The factors not included in each graph are maintained at the central value of the central composite design (t = 5 min in graph (a) and NaCl = 5 % in graph (b))

As there was a linear relationship between NaCl concentration with the a_w of simulated meat samples, regression modelling was also conducted to establish a relationship between a_w and *L. monocytogenes* inactivation values. It is important to highlight in this study that the addition of 5 % glycerol exert an additional effect on reducing a_w values of samples. The results of the regression modelling, proceeded by the backward stepwise regression procedure ($p \leq 0.05$) are shown in Table 3.4, and the polynomial model resulted is shown as Equation 3.2.

$$\log\left(N/N_0\right) = 118.65831 - 125.38578 \cdot a_w - 4.01007 \cdot \log t + 3.98870 \cdot (\log t)^2 - 0.20832 \cdot P + 0.21577 \cdot a_w \cdot P - 0.00701 \cdot P \cdot \log t \quad \text{Eq. (3.2)}$$

where $\log(N/N_0)$ is the logarithmic reduction of *L. monocytogenes*; a_w is the water activity of simulated meat samples; P is the applied pressure (MPa); and *t* is the pressure-holding time (min).

Table 3.4- Results of the multivariate regression describing the effect of a_w, pressure and pressure-holding times on *Listeria monocytogenes* inactivation due to HHP processing of simulated meat medium

Terms	Regression coefficients	Standard Error	*t*	*p*
Intercept	118.658	8.001	14.831	0.000
a_w	-125.386	8.451	-14.836	0.000
log t	-4.010	0.752	-5.333	0.000
(log t)^2	3.989	0.266	15.012	0.000
P	-0.208	0.018	-11.803	0.000
a_w ·P	0.216	0.019	11.573	0.000
t ·P	-0.007	0.001	-4.635	0.000

[a] Only the statistically significant terms were kept in the model through the backward stepwise regression.

This model is also statistically significant (*F*–value = 128, $p \leq 0.0001$), with goodness-of-fit R^2_{adj} = 0.91 (R^2 = 0.92). The lack of fit of the model was non-significant (*F*–value = 7.62, $p > 0.05$). This equation would be useful to obtain and compare estimates of the inactivation of *L. monocytogenes* based a_w data from meat products, as many authors have considered the a_w an independent variable when modelling pressure-induced inactivation (Bover-Cid et al., 2015, 2017; Rubio et al., 2018). It is important to highlight that the significant first order and second

order terms of the variables pressure and pressure-holding times on Equation 3.2 remained the same as in Equation 3.1. The inactivation pattern of *L. monocytogenes* described by this model is also in accordance with other studies, where the pressure-lethality is reduced by decreasing the a_w of meat products (Hayman, Kouassi, Anantheswaran, Floros, & Knabel, 2008). The effects of pressure and pressure holdings-times on inactivation trend are the same as in Equation 3.1.

The performance of Equation 3.1 for predicting the level of *L. monocytogenes* inactivation was assessed by calculating the indexes A_f and B_f based on the experimental data and on model predictions. The A_f and B_f values determined for the polynomial model generated were 1.06 and 1.04, respectively. The model predictions present a perfect match with the observed data when $A_f = B_f = 1$, which would be the ideal case (Baranyi et al., 1999). The A_f determined in this study is acceptable considering that for each model variable, A_f typically increases by 0.1 to 0.15 (Ross, Dalgaard, & Tienungoon, 2000). The B_f value indicates that overall the model underestimates the inactivation that really occurred during HHP processing in about 4 %. Regarding Equation 3.2, the model with a_w as independent factor was even more accurate in predicting the inactivation of *L. monocytogenes,* underestimating the observed inactivation data in only 2 %, with $A_f = 1.05$ and $B_f = 1.02$.

The models developed in this study, along with supporting external validation data obtained on RTE meat products can represent important tools to establish appropriate processing criteria and the effective application of HHP technology in meat industry. As it was already proven that the a_w plays an important role on the lethality of HHP processing in foods, a reliable characterization of this physicochemical parameter in meat products is essential to yield more accurate predictions of *L. monocytogenes* inactivation.

This modelling approach was conducted in order to quantify and evaluate the impact of the characteristics of a simulated meat medium and the technological parameters on the antilisterial effect of HHP treatments. Modelling studies such as the ones conducted by Valdramidis et al.

(2015) and Koseki et al. (2007) also evaluated the recovery of *L. monocytogenes* cells during the storage of simulated meat media and meat products previously submitted to HHP processing. Although high pressure application could result in undetectable levels of the pathogen immediately after the treatments, in some cases the injured cells can recover and grow in ready-to-eat meat products during shelf-life (Hereu, Dalgaard, Garriga, Aymerich, & Bover-Cid, 2014; Jofré, Aymerich, Bover-Cid, & Barriga, 2010; Myers, Montoya, Cannon, Dickson, & Sebranek, 2013; Valdramidis et al., 2015). Moreover, cells that resisted pressure treatments could grow with increased resistance to subsequent stresses in foods (van Boeijen et al., 2010). Thus, the possible recovery of sublethal injured cells in foods submitted to pressure treatments should be also considered to guarantee their safety and microbiological criteria compliance.

3.4. Conclusions

The results obtained in this study highlight the impact of the a_w, which is directly related to the sodium chloride concentration of the simulated meat evaluated, as a relevant intrinsic factor on the effectiveness of high-pressure processing technology application to inactivate bacteria. The decrease in pH and increase in sodium nitrite concentration of simulated meat medium did not potentiate the lethality of high-pressures. On the other hand, sodium chloride showed a strong interaction with pressure intensities on inactivating *L. monocytogenes*, which shows that food components/additives and technological parameters can simultaneously influence on pressure-induced inactivation. Once validated in meat products, the models developed in this study enable to define the pressure and pressure holding-times required to meet a given target of *L. monocytogenes* inactivation as a function of its sodium chloride concentration or a_w.

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Chapter IV

Chapter IV: Model for *Listeria monocytogenes* inactivation by high hydrostatic pressure processing in Spanish chorizo sausage

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4.1. Abstract

A central composite design was implemented to study the effect of three factors on HHP-induced *L. monocytogenes* inactivation in Spanish chorizo sausage, in order to increase its effectiveness: product a_w (0.79- 0.92), pressure intensities (349-600 MPa, at 18 °C) and holding time (0-12.53 min). Response surface methodology was implemented with backward stepwise regression to generate a model that best fitted to the experimental data. All the three factors studied significantly influenced HHP inactivation of *L. monocytogenes* ($p < 0.05$). Pathogen reductions increased as the pressure and duration of HHP treatments rose. Low values of a_w seemed to exert a protective effect on *L. monocytogenes* and a pressure of 400 MPa did not lead to significant pathogen reductions. The model was validated with independent published data. Accuracy and bias factors were also determined to evaluate the performance of the developed model, which was considered acceptable for prediction purposes. The model generated represents a mathematical tool that will help food manufacturers improve the efficacy of HHP processing of chorizo sausage and observe the regulatory authority's specifications regarding *L. monocytogenes* levels while maintaining food safety.

Keywords: modelling, ready-to-eat products, high-pressure processing, response surface methodology

4.2. Introduction

Chorizo is a typical Spanish dry-fermented sausage, manufactured using traditional technologies and composed of meat and fat, together with salt, garlic, Spanish paprika and oregano. In industrial formulations curing agents such as nitrate or nitrite are usually added in order to inhibit the growth of undesirable bacteria and promote colour formation (Sidira et al., 2015). Nevertheless, because of the negative health image of nitrite in meat products, it is now becoming more common to manufacture chorizo sausage without this additive. However, the addition of nitrite to meat products increases lag-phase duration and slows the growth of pathogens such as *Listeria monocytogenes* (Xi et al., 2011). Therefore, from a safety point of view, the absence of nitrate or nitrite is of concern in relation to the control of *L. monocytogenes* in the meat industry (Hospital et al., 2012).

Research has indicated that *L. monocytogenes* can contaminate raw ingredients (De Cesare et al., 2007; Thévenot et al., 2005), is able to survive until the end of the manufacturing process (Drosinos et al., 2006) and thus may not be completely eliminated during the production of dry-fermented sausages (Lindqvist and Lindblad, 2009). Consequently, the presence of *L. monocytogenes* in fermented meats means that more measures must be taken to avoid pathogen growth.

High hydrostatic pressure (HHP) has primarily been used to improve the microbiological safety and shelf-life of ready-to-eat (RTE) meat products as a novel pre/post-packaging non-thermal decontamination technology in the meat industry (Bajovic et al., 2012). Several studies have focused on the application of HHP to control *L. monocytogenes* present in different meat products (Ananou et al., 2010; Balamurugan et al., 2016; Bover-Cid et al., 2015; Hereu et al., 2014, 2012; Patterson et al., 2011; Porto-Fett et al., 2010; Valdramidis et al., 2015). In general, pathogen lethality during HHP treatment depends on various processing parameters such as the pressure level and holding time. Aymerich et al. (2005) and Jofré et al. (2009) have reported that pressure treatments of up to 300 MPa are insufficient to inactivate *L. monocytogenes* in different meat products. Regarding time, the meat industry currently applies the shortest HHP

treatment on production lines, from 3 to 6 min maximum (Garriga et al., 2004; Tonello, 2011).

In addition to processing parameters, intrinsic factors of the food matrix also exert a dramatic effect on bacteria inactivation during pressure treatment (Alpas et al., 2000; Patterson, 1999; Smelt, 1998). It is known that a low water activity (a_w) protects microorganisms against the effects of pressure (Patterson, 1999; Smelt, 1998). To date, no studies have been carried out considering microbial inactivation by HHP treatment of the same type of fermented meat product with different values of a_w.

In this respect, it should be noted that the a_w of commercial chorizo sausage can vary widely, from 0.79 to 0.94, as has been reported in the literature for different varieties of this meat product (Astiasaran et al., 1990; Gómez and Lorenzo, 2013; Pérez-Casas et al., 1999; Salgado et al., 2006). Thus, it is necessary to determine adequate pressure processing parameters and characterise product a_w in order to ensure that optimum processing conditions are selected for HHP treatment of dry fermented sausages.

The aim of this study was to obtain a model of *L. monocytogenes* inactivation based on pressure and pressure-holding time, which are the most important HHP technological parameters, and the a_w as the main intrinsic factor in traditional dry chorizo sausage (produced without the addition of nitrate or nitrite).

4.3. Material and Methods

4.3.1. Experimental design

A central composite design (CCD) was implemented in order to study the effect on *L. monocytogenes* inactivation of the following factors: meat product water activity (a_w), HHP treatment pressure (P) and HHP treatment time (t). The five levels of the three factors are shown in Table 4.1. A total of 16 experiments were performed in random order (trial order) because randomisation allows the experimenter to avoid erroneous conclusions due to extraneous sources of variability (Joglekar and May, 1987; Robinson, 2000).

Table 4.1- Three selected factors (independent variables) and experimental ranges considered for each factor according to a central composite experimental design.

Factors	*Levels* [a]				
	-1.68	***-1***	***0***	***+1***	***+1.68***
a_w	0.79	0.82	0.86	0.90	0.92
P (MPa)	349	400	475	550	600
Time (min)	0	2.5	6.25	10	12.53

[a] Considering the circumscribed central composite experimental design for three factors, the scaled value for α relative to the coded values ± 1 is 1.68 ($2^{3/4}$).

In this type of experimental design the central points are duplicated in order to evaluate experimental error and thus lack-of-fit of the model.

4.3.2. Bacterial strains and culture preparation

For inoculation, a four-strain cocktail mixture of *L. monocytogenes* was used. Three strains of *L. monocytogenes* were isolated from dry-fermented meat products and the fourth was obtained from the Spanish Type Culture Collection (CECT935, serotype 4b).

To prepare the inoculums, *L. monocytogenes* cultures were grown individually. Initially, 100 µL of the stock cultures (stored in 20 % glycerol at -80 °C) was transferred to tubes containing 10 mL of Brain Heart Infusion broth (BHI, Scharlau, Barcelona, Spain) and incubated for 24 h at 37 °C. Subsequently, 1 mL from each individual strain culture was transferred to a second tube containing 9 mL of BHI and incubated overnight for 18 h at 37 °C, yielding early stationary phase cultures. The inoculation cocktail was prepared by mixing equal volumes of the four individual cultures in 0.1 % peptone water in a sterile container in order to obtain a level of inoculum of about 10^6 cfu/g of sausage mixture.

4.3.3. Meat product and sample preparation

All the sausages used in this study were manufactured on the same day, using the same technology and according to a traditional formulation, which consisted of 70 % pork meat and 30 % pork back fat. Lean pork meat and pork back fat were minced (P-32 FUERPLA, Valencia, Spain) to a particle size of about 15 mm and subsequently mixed in a vacuum mixer (A-85

FUERPLA, Valencia, Spain) with the following common ingredients per kilogram of meat mixture: 20 g sodium chloride, 20 g paprika, 10 g dextrose, 1.5 g garlic, 1.0 g oregano, 1.0 g black pepper and 1.0 g polyphosphates. Then the cocktail cultures were added and mixed for 1 min. This sausage mixture was stuffed into natural casings (62–65 mm ø) in pieces weighing 800–900 g. The sausages were fermented and dried in a drying chamber (Hermekit, Cenfrio, Spain) at 15 °C and 90–100 % relative humidity (RH) for 18 h, at 22–23 °C and 90 % RH for 48 h, and at 14–15 °C and 80–90 % RH for 10 days. Then, the RH was reduced 5% per week until reaching 75 %. These conditions were maintained until the end of the ripening process.

To adjust the a_w of the sausages in accordance with the CCD, weight losses and a_w were evaluated throughout the process of ripening. The a_w was measured using a Decagon CX-2 AQUALAB hygrometer (Decagon Devices Inc., Pullman, WA, USA) at 20 ºC. Besides, as control of the drying process, the pH values were determined by puncture with a pH meter model 507 (Crison Instruments, Barcelona, Spain). In all sausages manufactured, the pH value (pH = 4.8-5.3) was within the range of common values for this product.

Sausages with the desired a_w were packed in plastic bags (polyamide/polyethylene with an oxygen transmission rate of 30–40 cm^3/m^2/24 h/bar, at 23 °C and 50 % RH and a water vapour transmission rate of 2.5 g/m^2/24 h at 23 °C and 50 % RH, supplied by WK Thomas España S.L., Rubí, Spain) and vacuum sealed using a packer (mod. EVT-7-TD Tecnotrip, Barcelona, Spain).

4.3.4. High-pressure processing

Packaged samples were subjected to HHP according to the CCD, i.e. in the range of 349–600 MPa for 0–12.53 min. HHP treatments were performed in an industrial hydrostatic pressure unit (Wave 6000/135. NC Hyperbaric, Burgos, Spain) equipped with a 135 L high-pressure vessel using additive-free water as the pressure transmitting fluid. In all cases, the initial water temperature was 18 °C, the treatment pressure was reached in approximately 4 min and decompression was instantaneous.

4.3.5. Microbiological analysis

L. monocytogenes counts were monitored prior to and after HHP treatments of the chorizo sausages. Bacterial inactivation was evaluated in terms of logarithmic reductions as the difference between counts after the treatments (N, log cfu/g) and the initial inoculum level (N_0, log cfu/g) (i.e. $log\ (N/N_0)$). For each combination of the CCD, HHP treatments were replicated. Thus, one day, all combinations of factors considered were performed using two sausages (control- N_{01} and treated- N_1) per trial. The next day, the 16 combinations of the CCD were made again using two other different sausages (control-N_{02} and treated-N_2). For each trial the inactivation value was calculated as the mean value of inactivation value 1 (calculated as log N_1/N_{01}) and the inactivation value 2 (calculated as log N_2/N_{02}).

For microbiological determinations, the sausages were sampled by aseptically opening the casings with a sterile lancet and removing 10 g from different parts along the sausage. Samples were placed in a sterile plastic bag, mixed (1:10) with buffered peptone water (Scharlau, Barcelona Spain) in a PK 400 Masticator (IUL, S.A., Barcelona, Spain) for 2 min and then incubated for 1 h ± 5 min at 20 °C ± 2°C. The homogenate was serially diluted in sterile tryptone water (Scharlau, Barcelona Spain), plated onto the selective media ALOA® (Biomerieux, Madrid, Spain) and incubated at 37 ºC ± 1°C for 48 h ± 3 hours (ALOA® COUNT Method, AES 10/05-09/06).

4.3.6. Mathematical modelling

Response surface methodology (RSM) was implemented to study the relationship between the three independent variables (a_w, P and t) and *L. monocytogenes* inactivation by HHP. Analysis of variance (ANOVA) and LSD (least significant difference) *post* hoc tests were carried out to compare *L. monocytogenes* inactivation results ($p < 0.05$). In order to generate a second order polynomial equation that best fitted to the experimental data, a backward stepwise regression method was conducted, using STATISTICA® software, version 10 (Statsoft, Portugal). Only the statistically significant terms were retained in the final equation ($p < 0.05$). The statistical

significance of the model was evaluated using the model *F*-value and lack-of-fit value. The adjusted R^2 goodness-of-fit index (R^2_{adj}) was evaluated to determine how well the model fitted to the experimental data. Response surfaces were drawn to illustrate the effect of a_w, *P* and *t* on *L. monocytogenes* inactivation.

4.3.7. Model validation

The accuracy factor (A_f) and bias factor (B_f) were calculated according to Equations 4.1 and 4.2 (Baranyi et al., 1999). These values can be considered measures of the performance of predictive models in risk assessment (Ross et al., 2000). The A_f indicates the spread of results in comparison with the model predictions, while the B_f is a measure of the extent to which the model under- or overestimates the inactivation observed. A_f and B_f values closer to 1 indicate a better agreement between the experimental data and the model predictions.

$$A_f = 10^{\frac{\sum|\log(predicted/observed)|}{n}} \quad \text{Eq. (4.1)}$$

$$B_f = 10^{\frac{\sum\log(predicted/observed)}{n}} \quad \text{Eq. (4.2)}$$

where *n* represents the number of trials.

Data from the international scientific literature regarding HHP-induced *L. monocytogenes* inactivation were considered for model validation.

4.4. Results and Discussion

4.4.1. Inactivation of *L. monocytogenes* by HHP

Table 4.2 shows the results for *L. monocytogenes* inactivation expressed as $log\ (N/N_0)$ for all the CCD combinations of a_w, *P* and *t* tested. The reduction in *L. monocytogenes* viability after HHP treatment ranged from no observed reduction to 4.31 log cfu/g, depending on the trial.

Table 4.2- Results of *Listeria monocytogenes* inactivation after high hydrostatic pressure processing for the combinations of factors considered in the central composite design.

Trial	*Run*	a_w	*Pressure (MPa)*	*Time (min)*	*Inactivation log (N/N_0)*[1]
1	11	0.82	400	2.50	0.44 (0.28/0.59)[a]
2	3	0.82	400	10.00	-0.11 (-0.20/-0.01)[a]
3	9	0.82	550	2.50	-1.21 (-1.00/-1.41)[b]
4	15	0.82	550	10.00	-2.49 (-2.68/-2.29)[c]
5	8	0.90	400	2.50	-0.25 (0.17/-0.66)[a]
6	4	0.90	400	10.00	-1.27 (-0.95/-1.58)[b]
7	1	0.90	550	2.50	-1.69 (-1.64/-1.73)[bc]
8	10	0.90	550	10.00	-3.71 (-3.11/-4.31)[d]
9	12	0.79	475	6.25	-0.07 (0.02/-0.16)[a]
10	5	0.92	475	6.25	-2.17 (-2.37/-1.97)[c]
11	14	0.86	349	6.25	-0.04 (0.10/-0.17)[a]
12	7	0.86	600	6.25	-2.47 (-1.79/-3.15)[c]
13	2	0.86	475	0.00	0.00 (0.00/0.00)[a]
14	13	0.86	475	12.53	-2.50 (-2.31/-2.69)[c]
15	16	0.86	475	6.25	0.00 (0.00/0.00)[a]
16	6	0.86	475	6.25	-0.04 (-0.04/-0.04)[a]

[1] Mean of the inactivation values of two replicates (inactivation value 1/ inactivation value 2).
Values with different superscript letters are statistically different according to LSD *post* hoc tests ($p < 0.05$).

Maximum inactivation was achieved with the combination: $a_w = 0.90$, $P = 550$ MPa and $t = 10$ min (Trial 8, Table 4.2), which was statistically higher than the inactivation induced by HHP in the other trials ($p < 0.05$). In general, treatment lethality increased as treatment time and pressure rose, and with high a_w values.

Regarding treatment time, when the a_w was higher than 0.82, an increase in processing time improved *L. monocytogenes* inactivation by HHP. However, when the a_w was equal to 0.82, it was necessary to apply a pressure of 550 MPa to obtain a significant increase ($p < 0.05$) in the reduction of *L. monocytogenes* counts when the duration of HHP treatment increased (Trials 3 and 4).

As regards treatment pressure, increased pressure implied higher lethality of HHP treatments, irrespective of product a_w. Pressures equal or below 400 MPa did not lead to significant inactivation levels, findings also reported by Valdramidis et al. (2015) when applying pressures

of 450 MPa in a processed simulated cured meat for 3 min, and by Bover-Cid et al. (2011) when applying pressures lower than 450 MPa for longer periods (≤ 15.75 min).

Finally, considering different trials with the same levels of *P* and *t,* a significantly higher inactivation of *L. monocytogenes* was observed in products with a higher a_w when the duration of HHP treatment was longer than 2.5 min. For example, an increase in a_w from 0.82 to 0.90 led to a maximum inactivation of 1.27 log cfu/g at *P* = 400 MPa and *t* = 10 min (Trials 2 and 6); an increase in a_w from 0.79 to 0.92 led to a 2.17 log cfu/g inactivation t *P*= 475 MPa and *t*= 6.25 min (Trials 9 and 10); and an increase in a_w from 0.82 to 0.90 led to a 3.71 log cfu/g inactivation at *P* = 550 MPa and *t* = 10 min (Trials 4 and 8) Several studies have shown that *L. monocytogenes* baroresistance increases when meat products present a low a_w . Jofré et al. (2009), Porto-Fett et al. (2010) and Rubio et al. (2009) reported reductions of approximately 1 log cfu/g in *L. monocytogenes* counts when HHP treatments of 400-600 MPa were applied for 5–10 min to dry-fermented sausages with a_w ranging from 0.81-0.86. However, when similar HHP treatments were applied to dry-cured meat products with higher a_w values (0.89–0.92), reductions of 2–3 log cfu/g were obtained (Bover-Cid et al., 2011; Morales et al., 2006; Rubio et al., 2009).

In this study, none of the combinations of factors studied led to undetectable levels of *L. monocytogenes* in chorizo sausage. Bover-Cid et al. (2015) have reported complete inactivation (absence in 15 g/sample) of *L. monocytogenes* but only when pressures of 750 and 852 MPa were applied in dry-cured ham, i.e. pressure levels higher than those that can presently be achieved by industrial HHP equipment (600 MPa). In addition, the higher inactivation of the pathogen observed in the study by Bover-Cid et al. (2015), which varied between 0.92 and 6.82 log cfu/g when pressures ranging from 347 to 852 MPa were applied, can be attributed to the higher values of a_w of the dry-cured meat product studied, which varied from 0.86 to 0.96.

Similar results to those found in our study have been reported by Bover-Cid et al. (2011), Morales et al. (2006) and Rubio et al. (2009), who indicated that HHP treatments of 600 MPa/9 min, 450 MPa/10 min and 500 MPa/5 min, respectively, produced reductions in *L.*

monocytogenes counts of 2.9, 1.2–1.6 and 1.93 log cfu/g in dry-cured products (meat products with a_w values of around 0.90).

4.4.2. Regression modelling

The quadratic polynomial expression resulting from the statistical approach employed is presented as Equation 4.3, in which only significant terms have been included.

$$log(N/N_0) = -176.77051 + 394.94843 \cdot a_W - 237.76729 \cdot a_W^2 + 0.06104 \cdot P - 0.00007 \cdot P^2 + 0.54610 \cdot t - 0.02839 \cdot t^2 - 0.00078 \cdot P \cdot t \quad \text{Eq. (4.3)}$$

where *log (N/N$_0$)* represents the logarithmic reduction in *L. monocytogenes* (log cfu/g); a_w is the measured water activity of the chorizo sausages; *P* is HHP treatment pressure (MPa); and *t* is pressure-holding time (min).

The extent of inactivation was variable; however, all the three factors studied significantly influenced *L. monocytogenes* inactivation during HHP and are present in the model as linear and quadratic terms. The interaction between *P* and *t* was also significant, whereby an increase in pressure intensity increased the effect of holding time on *L. monocytogenes* inactivation, evidencing a synergistic effect of these technological parameters on HHP effectiveness. Pressure and time were the most important factors influencing *L. monocytogenes* inactivation by HHP in chorizo sausages, which is in accordance with a study by Rendueles et al. (2011), who have reported that the pressure applied and the holding time are the primary factors influencing the efficacy of HHP.

The *F*-value obtained for the model was 35.89, indicating that the model is significant ($p < 0.0001$), and an ANOVA revealed a satisfactory correlation between the regression model predictions and the experimental data, with an $R^2_{adj} = 0.88$ ($R^2 = 0.92$) for the dependent variable (i.e. *L. monocytogenes* inactivation) (Figure 4.1). The lack-of-fit value obtained was 1.27, indicating that lack of fit was not significant relative to the pure error ($p > 0.05$).

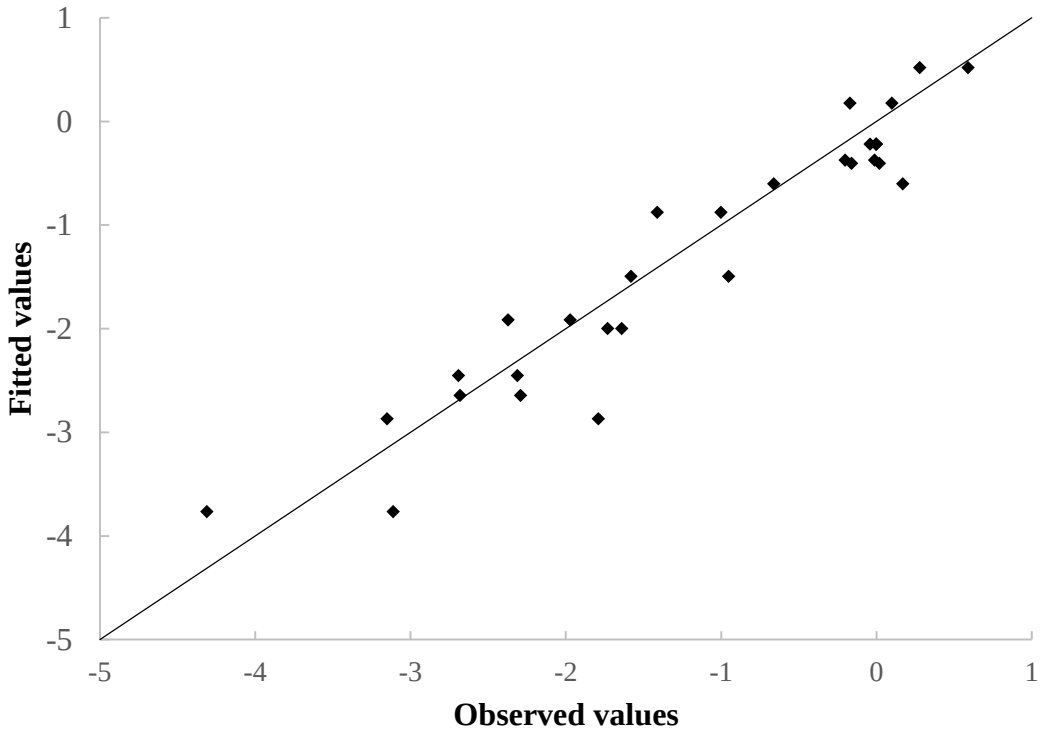

Figure 4.1- Predicted versus observed values for *Listeria monocytogenes* inactivation in chorizo sausage, following HHP treatments

The surface response graphs are shown in Figure 4.2. The surfaces were generated based on the polynomial equation developed (Equation 4.3) and provide an overview of how the three factors studied influenced HHP-induced *L. monocytogenes* inactivation. The curvature of the surfaces is attributed to the second order terms of the three factors evaluated. Both the increase in chorizo a_w and the increase in pressure values led to an increase in *L. monocytogenes* inactivation levels during HHP treatments (Figure 4.2a). In addition, longer HHP treatments in combination with higher intensity of pressures led to higher inactivation levels of *L. monocytogenes* in chorizo sausage (Figure 4.2b).

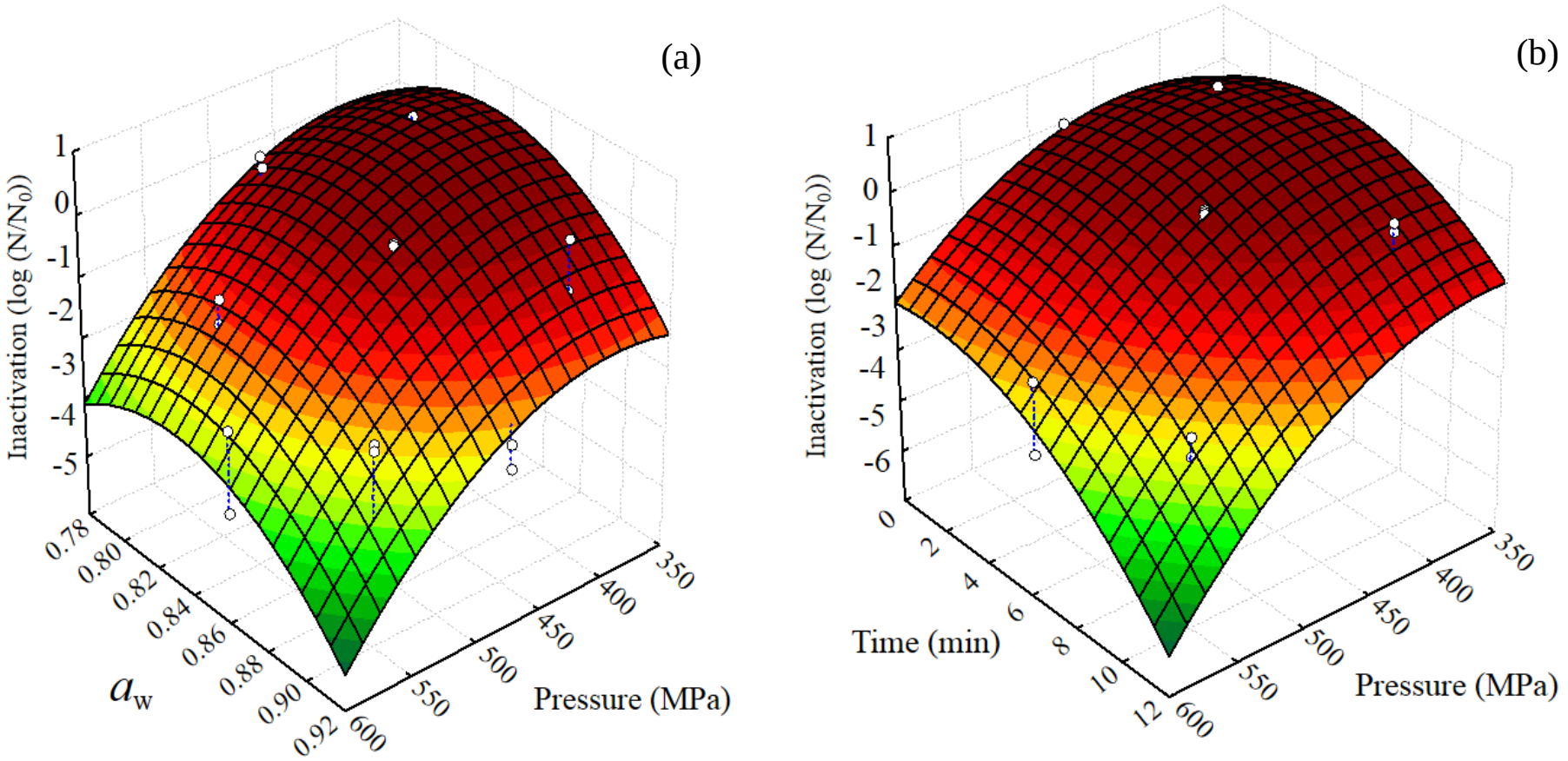

Figure 4.2- Response surface graphs of HHP-induced *Listeria monocytogenes* inactivation in chorizo sausage, according to the developed model. (a) a_w and P effects; (b) t and P effects. The factor not included in each graph is maintained at the central value of the central composite design (t = 6.25 min in graph (a) and a_w = 0.86 in graph (b))

4.4.3. Model validation

Based on the experimental data and on model predictions, the validation indexes (A_f and B_f) were determined according to Equations 4.1 and 4.2. The A_f and B_f values calculated for the polynomial model developed were 1.45 and 1.32, respectively. In an ideal case, $A_f = B_f = 1$ and the model predictions present a perfect match with the observed data. For each model variable, A_f typically increases by 0.1 to 0.15 (Ross et al., 2000), so an acceptable A_f value is expected to range between 1.3 and 1.5, which is consistent with the value obtained in the present study employing three independent variables (a_w, P and t). The B_f of the model was above one, indicating that overall the model yields "fail-safe" predictions of *L. monocytogenes* inactivation in chorizo sausage, i.e. it underestimates the inactivation that really occurs during HHP processing in about 32 %. However, B_f values ranging from 0.87–0.95 or 1.11–1.43 are considered acceptable (Ross, 1999).

To date there is no modelling study on pressure-induced inactivation of *L. monocytogenes* on fermented meats, under the conditions tested in this study. Comparisons between the predictions of the polynomial model generated in this study with literature models generated in meat products were conducted in order to evaluate the possibility of the existence of a general model to describe the inactivation of *L. monocytogenes* by HHP in these types of foods.

The model developed in this study was satisfactorily adjusted to the data published by Bover-Cid et al. (2015) for dry-cured ham, considering pressure intensities (600 MPa), treatment time (5 min) and product a_w values (0.86-0.92) within the interval ranges considered in this study. The $A_f = 1.09$ and $B_f = 0.97$ indicated a good fit of the model to the dataset, although in this case it gave slightly "fail-dangerous" estimates. It is important to highlight that only data obtained for dry-cured ham with similar characteristics of the chorizo sausage samples, regarding fat and salt content and a_w, were considered for validation, thus there were no large deviations between predicted and observed values.

Overall, the inactivation models on meat products currently available in literature overestimate the inactivation that was observed in this study. For instance, the HHP inactivation model of

Hereu et al. (2012), obtained in challenge studies with mortadella, was not adequate to describe our experimental data, although mortadella is also a product with high fat content. The inactivation predicted by their model considering a pressure intensity of 550 MPa and pressure holding time of 10 minutes was -6.27 log cfu/g, while in this study at the same conditions the inactivation predicted by our model was -3.76 log cfu/g. Furthermore, the model for *L. monocytogenes* inactivation on dry-cured ham by HHP, developed by Bover-Cid et al. (2011), which considered the technological parameters pressure, time and temperature as independent variables, overestimated the inactivation that really occurred in Spanish sausage in about 8 % (B_f = 0.92). Although in this case the B_f was within the acceptable range proposed by Dalgaard (2000), 0.75-1.25, the A_f = 2.44 was higher than the upper limit proposed (1.60), considering an increase of 0.15 for each variable of the model.

Many authors have demonstrated that the intrinsic characteristics of food matrices can interfere considerably on the HHP efficacy to inactivate pathogenic bacteria in foods (Georget et al., 2015; Syed et al., 2016). Thus, for reliable application, models should be developed with a product-oriented approach, taking into consideration the specific characteristics of a food commodity (Bover-Cid et al., 2015; Georget et al., 2015).

4.4.4. Model application

Although HHP has been extensively implemented in the food industry, the effect of intrinsic factors of specific food matrices on its efficacy should be further investigated and modelled. In this study, the influence of the a_w of chorizo sausages, an intrinsic factor, was considered together with the influence of technological parameters. This product-oriented approach can help food managers and manufacturers to simulate and optimise HHP technology conditions in order to observe or establish Food Safety Objectives (FSO), increasing processing efficacy while reducing technological costs. For instance, according to European Commission Regulation N° 2073/2005 (European Comission, 2005), food manufacturers should ensure that *L. monocytogenes* levels do not exceed 10^2 cfu/g throughout the shelf-life of RTE products.

The Spanish Agency of Food Safety has established that food manufacturers should ensure a 4 log reduction in *L. monocytogenes* in RTE meat products (AESAN, 2005). In the hypothetical case of a batch of chorizo sausages with a mean a_w = 0.86 subjected to HHP, if the pressure applied by the equipment was set at 550 MPa, a treatment of at least 12.3 min would be required in order to observe these criteria, according to the empirical equation generated. If we locate the pressure and time values (550 MPa, 12.3 min) on the contour plot of Figure 4.3, where a_w was set at 0.86, it is possible to confirm that the desired reduction would be achieved. It is important to highlight that the model is applicable only in the ranges set in the experimental design for the independent variables.

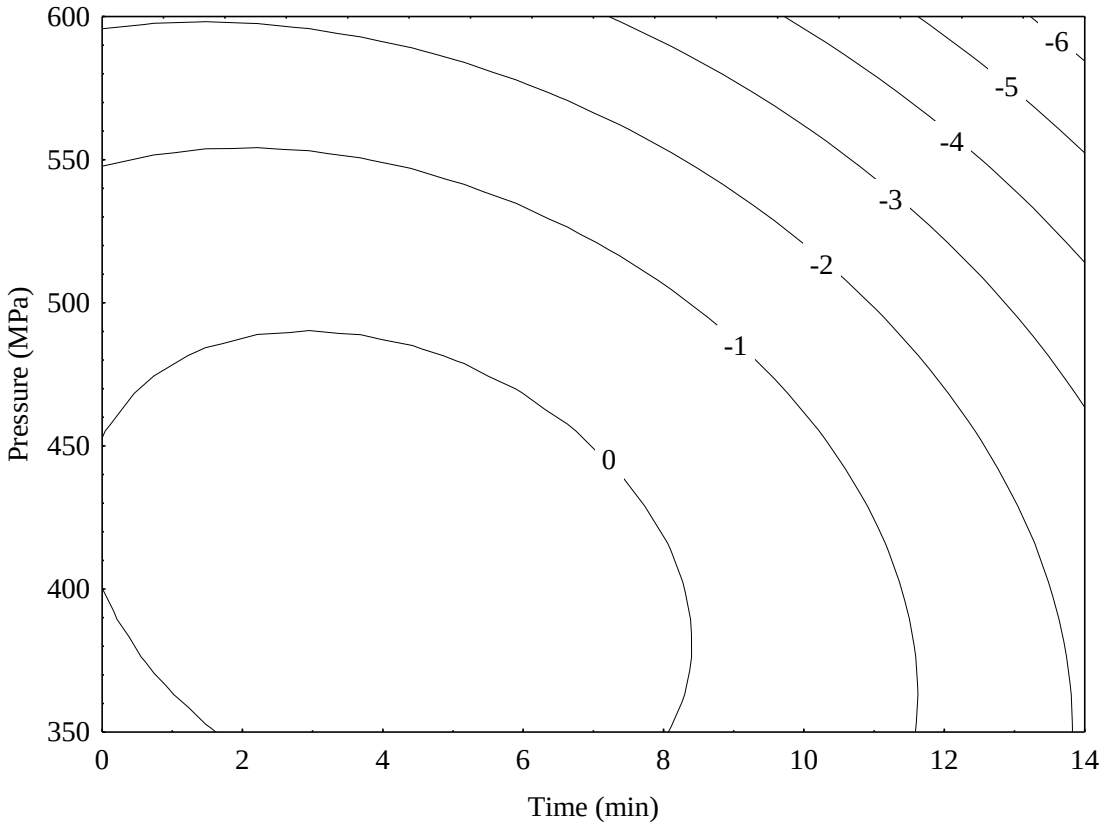

Figure 4.3- Contour plot describing HHP-induced *Listeria monocytogenes* inactivation (log cfu/g) in chorizo sausages with a a_w = 0.86, as a function of pressure intensity (MPa) and time (min)

Although HHP can reduce *L. monocytogenes* levels, further studies are required to evaluate and model the behaviour of this pathogen during chorizo sausage shelf-life, taking also into consideration the recovery of pressure-injured cells.

4.5. Conclusions

The a_w, pressure and time of HHP significantly affected the reduction in levels of *L. monocytogenes* in chorizo sausage, as demonstrated in this modelling approach. The results obtained in this study reinforce the need of product-oriented approaches when modelling the HHP inactivation of *L. monocytogenes* in meats, since the intrinsic characteristics of food matrices such as a_w, as well as their composition, in combination with the technological parameters (pressure, time, temperature, etc.), can influence on its efficacy. The model developed in this study can help food manufacturers to optimise and manage HHP treatments in order to ensure Spanish chorizo sausage safety in accordance with the regulations established for RTE meat products with respect to *L. monocytogenes* levels.

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Chapter V

Chapter V: Food Risk Assessment framework: Foundations and concepts

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5.1. Abstract

The risk analysis paradigm creation was encouraged by food safety issues derived from the globalization of trade, which includes the transmission of harmful resistance bacteria along the food chain and the presence of pesticides in foods. Risk assessment is the scientific component of the risk analysis process, and can be defined as the qualitative and/or quantitative evaluation of the adverse effects linked to chemical, physical and biological agents that may be present in foods. In this chapter, the main concepts and foundations of the risk assessment in foods are presented. The limitations and challenges of a risk Assessment and current developments are also described.

Keywords: risk analysis, microbial risk assessment, chemical risk assessment, food safety.

5.2. Introduction

Globalization of trade is having a big impact on food systems worldwide, resulting in greater food availability and diversity. A food commodity produced in one side of the world can be available in the other side in a matter of days. The increase in food productivity is driven by scientific and technological advances, genetic improvements, development of fertilizers and pesticides, use of antibiotics and growth promoting substances (Cummins 2017; FAO 2004). As consequences of the globalization of trade, the transmission of harmful bacteria with increased resistance and the presence of chemicals with toxicological effects are of big concern for human health. Thus, the reduction in barriers to the cross-border movement of foods has serious implications for food safety.

To face the issues resulted from globalization, the risk analysis approach has been created and is used as a dominant process to ensure food safety. Risk assessment is the scientific based component of risk analysis, and consists of a systematic framework conducted with the goal to achieve a full understanding of the nature, magnitude and probability of a potential hazard in foods (Kavlock et al. 2018).

A Microbial Risk Assessment (MRA) is performed to describe the risk and the potential adverse health effects of microbial hazards in the whole farm-to-fork food production chain or part that is relevant to the problem (Nauta 2008; Codex Alimentarius Commission 1999). Chemical risk assessments (CRA) can be described as the characterization of potential hazards and the associated risks to life and health resulting from exposure of humans to chemicals present in food over a specified period (EFSA, 2018).

The results of a risk assessment are an important management tool that can help in the detection of critical points in the food chain, in the assessment of interventions strategies and in the elaboration of standards for food in international trade (FAO/WHO 2008). In this chapter, the foundations and concepts of food risk assessments are described.

5.3. Hazard *versus* risk

Based on the need of uniform terminology, the Codex Alimentarius committee defined and published terms of risk assessment related to food safety, according to recommendations of Food Agriculture Organization (FAO)/World Health Organization (WHO) (FAO/WHO 2013). Among the definitions, the terms "hazard" and "risk" are fundamental, since in many languages these terms are not differentiated. According to Codex, a hazard is a biological, chemical or physical agent that can cause an adverse effect on heath, while risk is the probability of occurrence of an adverse health effect (i.e. death or illness) as a consequence of the presence of a hazard in foods (FAO/WHO 2013).

The definitions of hazard and risk published by the Codex, which cover chemical, biological and physical agents, differ from the definitions of bodies that deal specifically with CRA. In a CRA, the chemical is not the hazard by itself, but a property associated to it. According to the International Programme of Chemical Safety (IPCS), hazard is an "inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub) population is exposed to that agent". Finally, risk is defined as the "probability of an adverse effect in an organism, system, or (sub) population caused under specified circumstances by exposure to an agent" (IPCS 2004).

5.4. Risk assessment and its role in Risk analysis

The structural framework of risk assessments was formalized by the development and adoption of the risk analysis paradigm, by FAO/WHO taking the lead of World Trade Organization (WTO) in 1995 (Pérez-Rodríguez and Valero 2013). Risk analysis is a process comprising three components: risk assessment, risk management and risk communication (Figure 5.1) (FAO/WHO 2013). The development of food standards to ensure global food safety is based on the systematized risk analysis process (Cummins 2017; Pérez-Rodríguez and Valero 2013).

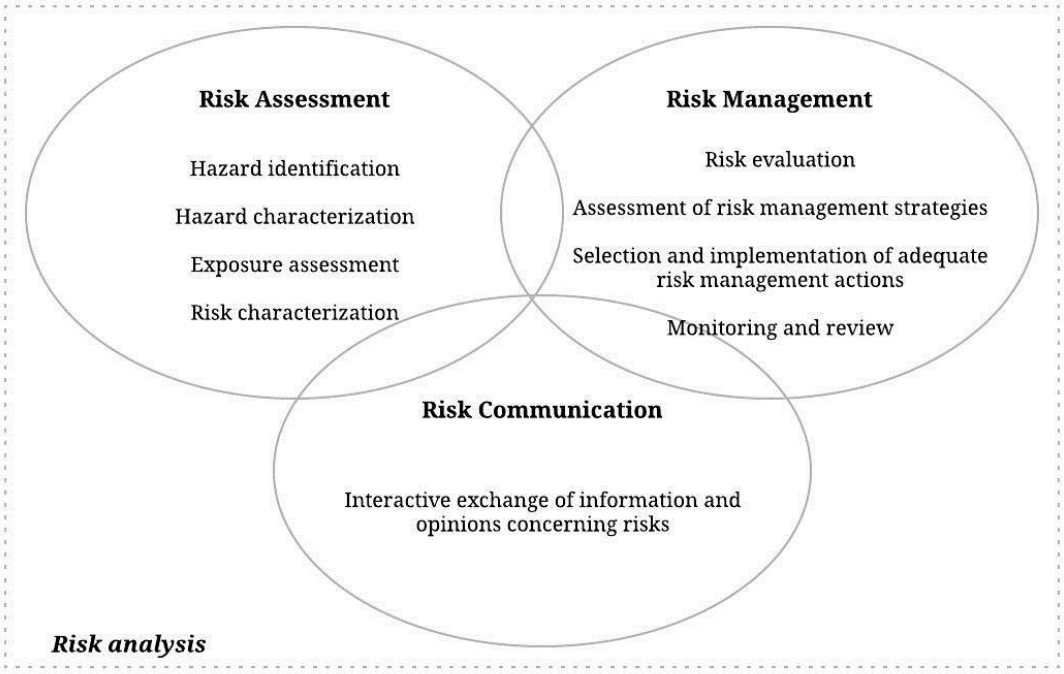

Figure 5.1- Interaction between the three components of risk analysis

Risk assessment, the central scientific component of the risk analysis process, is the qualitative and/or quantitative evaluation of the adverse effects linked to chemical, physical and biological agents that may be present in foods (FAO/WHO 2013). In the qualitative, risk is described by descriptive terms, while in a quantitative risk assessment the risk is estimated in terms of numerical outcomes, typically the probability of illness or death (Cummins 2017). Risk assessment was developed due to the need of making decisions to protect health in spite of scientific uncertainty (FAO/WHO 2009).

The decision on whether a risk assessment is necessary is taken by risk managers, which carry tasks including the description of the objective and the questions to be answered with the risk assessment. Risk managers also establishes the risk assessment policy, set time schedules and provide the resources needed for the risk assessment to be carried out (FAO/WHO 2009).

The risk management component of a risk analysis is in charge of deciding in whether a risk is acceptable in the light of the results of the risk assessment, and what control measures must be implemented in case the risk is no acceptable (FAO/WHO 2013). The risk management team

may be integrated by industry, public body representatives and policy makers alike (Cummins 2017).

Risk communication refers to the exchange of information and opinions regarding risk between risk assessors, risk managers and all stakeholders. This risk analysis component is critical to ensure that, regardless of scientific understanding, the aims and outcomes of a risk assessment are communicated to all the interested parties in a clear and effective manner (FAO/WHO 2013).

Although the interaction between the three risk analysis components is relevant, they may be functionally separated in order to avoid conflicts of interest or bias in the risk assessment process. Finally, the risk analysis process might be evaluated and reviewed when appropriate.

5.5. Risk assessment framework

The development of a risk assessment comprises four well established components of (i) hazard identification, (ii) hazard characterization, (iii) exposure assessment and (iv) risk characterization (CAC 1999). Although the same structure is adopted for microbial and chemical risk assessments, it is appropriate to subdivide their descriptions in individual sections, since some terms adopted for chemicals are different from terms adopted for microbial hazards. The definitions of risk assessment in the context of chemicals have been developed as a part of the project "Approaches to the Assessment of Risk from Exposure to Chemicals" (IPCS 2004), while the definitions for MRA are the ones established by the Codex Alimentarius (CAC 1999).

5.5.1. Microbial risk assessment concepts

Figure 5.2 depicts the four MRA components and briefly summarizes its main outputs and the type of information it describes. The MRA scope is defined between the risk question and the hazard identification.

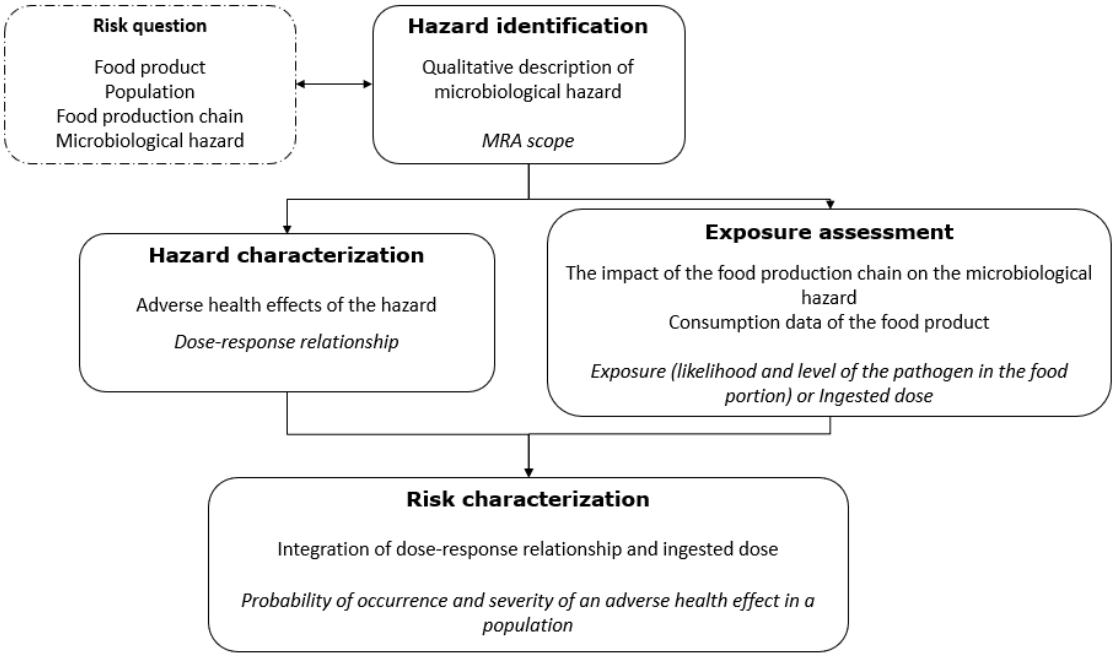

Figure 5.2- The main outputs (in italic) and the type of information described in each of the four components of a microbiological risk assessment

Similarly, the scope of the four components depends on the precise objective of the MRA. Generally, the objective is described as a risk question developed by risk managers in consultation with risk assessors. This question can describe the hazard, food, population and steps in the food production chain to be considered (Dennis et al. 2008; Nauta 2008). Two examples of risk questions from the literature are:

What is the efficacy of different intervention strategies to reduce the risk of acquiring *V. parahaemolyticus* gastroenteritis from raw oyster consumption? (FDA 2005)

What is the estimation of the number of listeriosis cases per year in the European Union population from consumption of a meal containing each of the three ready-to-eat (RTE) food categories: heat-treated meat, gravad and smoked fish and soft and semi-soft cheese? (Pérez-Rodríguez et al. 2017)

The hazard identification aims to identify microorganisms or microbial toxins of concern in the food product or water. It is predominantly a qualitative description of the microbiological

hazard of concern as well as relevant related data, such as clinical and surveillance data (FAO/WHO 2003; Lammerding and Fazil 2000).

The hazard characterization provides a qualitative and/or quantitative description of the adverse health effects that may result from ingestion of the microorganisms or microbial toxins. When quantitative data are available, a dose-response model is the main output of this component. The dose-response model describes the relation between the dose ingested (e.g. cfu/g or ml of a food product) and the frequency of a given effect (e.g. vomiting, or diarrhoea, or hospitalization, or death).

The exposure assessment provides a qualitative and/or quantitative estimate of the likelihood and level of the pathogen in a determined portion of food. Qualitative exposure assessments are descriptive or categorical treatments of information, whereas quantitative assessments are mathematical analyses of numerical data. If the available data are inadequate to develop a quantitative assessment, a qualitative assessment may be developed by assigning descriptive ratings of probability and severity such as 'negligible', 'low', 'medium' or 'high' to the exposure factors (FAO/WHO 2008). The use of models and data from the predictive microbiology (PM) reseach area is often deployed in quantitative exposure assessments. PM models describe mathematically the behaviour of microrganims over time and according to environmental factors (Tenenhaus-Aziza and Ellouze 2015).

In the last component, the risk is characterized by combining the exposure assessment and dose-response relation (Nauta 2008). In a MRA, the risk is the probability of occurrence and severity of known or potential adverse health effects in a given population over a given period (Codex Alimentarius Commission 1999).

It is important to highlight that the estimation of microbial concentration and prevalence in food products by the end of the production process or at the time of consumption is typically more relevant to the industry than the estimation of number of illness. Thus, QMEA (Quantitative Microbiological Exposure Assessments) are usually performed rather than QMRA (Quantitative Microbial Risk Assessments) (Membre 2016).

5.5.2. Chemical risk assessment concepts

Despite of being essential building blocks for foods, chemicals can have a variety of toxicological properties, some which can be harmful for humans healthy. CRA forms the foundation of regulatory decisions for a wide range of chemical substances, including the ones intentionally added or chemical residues that end up in foods by the end of the production process or the distribution chain (EFSA 2018).

The CRA structure provides mechanism to review all the relevant information necessary to estimate health outcomes in relation to the exposure to chemicals present in foods. The four steps of risk assessment for food chemicals are briefly described below.

Hazard identification: the purpose of hazard identification is the evaluation of the weight of evidence that a chemical can cause an adverse health effect according to data available on toxicity and mode of action. These data can come from observations in humans, domestic or laboratory animals or in vitro studies. From the observed data, the toxicity nature or the health effect and the affected organs or tissues are identified (FAO/WHO 2009). Hence, at this stage two primary questions must be answered: 1) what is the nature of any health hazard to humans an agent may pose? 2) Under which circumstances the identified hazard may be expressed?

Hazard characterization: after confirming a cause-effect relationship between the exposure to a chemical and the incidence of an adverse health effect, this relationship is qualitatively or quantitatively described at the hazard characterization stage, including a dose-response assessment where possible. At this stage, dose-response data derived from observations during in vivo or in vitro studies are essential. Based on these data, the effects of increasing the exposure to a chemical with the increase in incidence of the adverse health effect are characterized at this stage, as well as the first adverse effect resulted from an increase in dose or exposure, *i.e.* the critical effect (FAO/WHO 2009). Besides, the level of exposure to a chemical that do not produce appreciable health effects or health-guidance values such as the ADI (acceptable daily intake) for additives or residues or TDI (tolerable daily intake) for

contaminants are established. From these heath-guidance values, the maximum legally allowed concentrations of a chemical in food commodities are set (Brimer 2011).

Exposure assessment: according to IPCS (2004), exposure assessment is the "evaluation of the exposure of an organism, system, or (sub) population to an agent (and its derivatives)". The exposure assessment takes into account the occurrence and concentration of the chemical in the diet and food consumption data, to estimate average and high level daily intakes (FAO/WHO 2009).

Risk characterization: the information of the exposure assessment and the hazard characterization is integrated to estimate quantitatively or qualitatively the potential health risk associated to the human exposure to a chemical hazard present in food. Risk estimates are communicated to risk managers for decision-making, including the clear explanation of any uncertainties derived from the limitations in the risk assessment process (FAO/WHO 2009).

5.6. Deterministic *versus* Stochastic risk assessment

Risk assessment models can be characterized as deterministic or stochastic with regard to how input variables are handled (Vose 2008). In the first approach, point-estimate values are used to describe the variables of the model and only individual scenarios are analysed. Since the worst-case scenario is typically reflected, deterministic approaches are usually unrealistic or "overcautious" and the outcomes are not representative of real situations (Tennant 2012; Pérez-Rodríguez and Valero 2013). In the second approach, variables are defined with probability distributions that englobe all possible scenarios, taking into account uncertainty and/or variability in those variables (Cummins 2017). Hence, stochastic approaches are more realistic of real-life scenarios.

5.7. Uncertainty and variability in risk assessments

These components are related to the level of knowledge on risk model inputs. Briefly, uncertainty is the lack of knowledge, for instance, regarding a quantity (Membré and Boué 2018). As uncertainty is usually related to analytical limitations or low-precision of

measurement methods, it can be reduced by further study, for example by increasing the number of samples analysed or by improving measurement methods. The prevalence of a pathogen in a food commodity can be used to illustrate uncertainty: to ascertain prevalence with 100 % of certainty, a 100 % of the food products might be tested for the presence of the pathogen, which is not feasible. Hence, we have to rely upon prevalence data available to estimate the prevalence of the whole population, and the greater the number of samples the higher is our degree of certainty regarding the estimate. On the other hand, variability represents the true heterogeneity in a population (Membré and Boué 2018). For instance, the ability to metabolize or detoxify chemicals can vary from person to person. This variability is not reducible by further study since it is related to natural randomness.

5.8. Limitations and challenges of risk assessment in foods

A multidisciplinary team that supplies the variety of knowledge to handle the available scientific information is required to carry out a risk assessment. It includes professionals from different fields, such as microbiology, mathematics, epidemiology, toxicology, food technology, social sciences, among others (Membré and Boué 2018). The complexity derived from this multidisciplinary approach represents a big challenge when performing a risk assessment. The lack of guides or protocols to develop risk assessments and the lack of harmonization in vocabulary or terms employed are also big limitations of the field, since the employment of a common structure would be crucial to compare hazards, risks, management measures, etc., between autonomous regions and ideally between countries, and over time. Finally, practical guidelines to translate the risk-based food safety management for operational use, as well as instructional and training resources to assist in building skills for risk assessments must be created (Membré and Boué 2018).

5.9. Current developments and future perspectives

The incorporation of omics technology in the exposure assessment component will move towards the next generation of microbiological risk assessment. With this technology, the

behaviour of microorganisms to food preservation treatments and environmental conditions will be described with mechanistic cellular information (den Besten et al. 2017; Brul et al. 2012). Njage and Buys (2017) included the potential of gene transfer between strains into the exposure to *Escherichia coli* due to the consumption of lettuce. Fritsch et al. (2018) worked on the refinements of a *Listeria monocytogenes* QMRA by integrating genomic data and considering phenogenotype associations for the hazard properties such as the growth ability at low temperature and the virulence. In addition, the use of whole genome sequencing (WGS) has been frequently used to refine the hazard identification component of MRA (Membré and Guillou 2016).

The QMRA community has invested great efforts and time to develop a rich variety of data, databases, models and softwares (Membré and Guillou 2016; Tenenhaus-Aziza and Ellouze 2015). However, their reusability and the information exchange between the software and databases may currently be difficult and time consuming (Plaza-Rodríguez et al., 2017). This situation represents an obstacle for the performance of risk assessment using the most up to date knowledge. A recent initiative aims to establish a new community resource called Risk Assessment Modelling and Knowledge Integration Platform (RAKIP). This platform will facilitate the sharing and execution of curated QMRA and PM models using a harmonized metadata schema and information exchange format. The aim of RAKIP is to promote knowledge reusability and high-quality information exchange between stakeholders within the QMRA and PM modelling (Haberbeck et al. 2018; Plaza-Rodríguez et al. 2017).

The approaches of chemical and microbiological risk assessment and the nutritional aspects of food consumption are integrated in one of the most recent risk-based method, the so-called risk-benefit assessments (RBA). Currently, most of the RBA integrate chemical and nutritional assessments, and microbial risk is occasionally assessed and mostly qualitatively (Boué et al. 2015). Some recent examples are the studies of Berjia et al. (2012) that integrated microbiological risks and nutritional benefits in cold smoked salmon and Boué et al. (2017) that integrated microbiological and chemical risks with nutritional benefits in infant feeding.

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Chapter VI

Chapter VI: High hydrostatic pressure of sliced fermented sausages: a quantitative exposure assessment for *Listeria monocytogenes*

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6.1. Abstract

Fermented sausages have traditionally been considered to be safe products from a microbiological point of view, mainly due to nitrite addition, their low a_w and pH. However, post-process contamination during slicing and packaging operations may increase microbial concentration and prevalence on final products. A stochastic simulation modelling approach was conducted to determine the extent of *Listeria monocytogenes* survival on sliced fermented sausages submitted or not to high hydrostatic pressure (HHP) treatments after post-process contamination (*i.e.*, cross-contamination during slicing). A probabilistic model comprising nine steps from mixing of raw materials to consumption was constructed. The effects of various HHP treatments and nitrite concentrations on *L. monocytogenes* distribution were assessed by means of the application of inactivation models, literature information and data obtained experimentally. Once implemented, the probabilistic model was simulated by using Monte Carlo analysis. The probability distribution of *L. monocytogenes* contamination levels was determined for various scenarios. Model outputs showed that cross-contamination during slicing was an important source contributing to increase pathogen prevalence and concentration on final products. Under all simulated scenarios, formulation and storage conditions, the level of *L. monocytogenes* on sliced vacuum-packed chorizo at the consumption phase was estimated to be low, although food safety was increased by pressure-treatments. Overall, the probabilistic model developed in this study from raw material reception up to the end of the shelf-life of sliced fermented sausages is proposed as a suitable tool to determine combinations of HHP treatments and nitrite concentrations ensuring the compliance with microbiological criteria.

Keywords: probabilistic model, cross-contamination, high-pressure, risk assessment, ready-to-eat meat, nitrite reduction.

6.2. Introduction

Chorizo is a Spanish dry-fermented sausage, produced from raw minced pork and fat seasoned with salt, Spanish paprika, garlic and oregano (Rubio, Possas, Rincón, García-Gímeno, & Martínez, 2018; Stollewerk, Jofré, Comaposada, Ferrini, & Garriga, 2011). This traditional product is characterized by a great diversity in methods of production, such as fermentation and drying practices and the addition of starter cultures, which results in more than 20 varieties of chorizo that differ in size and sensory characteristics (González & Díez, 2002; Leroy, Lebert, & Talon, 2015). Nowadays, it is usually encountered sliced and vacuum packed due to marketing, convenience and quality reasons (Stollewerk et al., 2011). The microbiological stability of chorizo depends on the combined effect of individual hurdles, including low pH and water activity (a_w) and the presence of curing salts, which could inhibit undesired microorganisms (Menéndez, Rendueles, Sanz, Santos, & García-Fernández, 2018). Although traditionally recognized as safe, this product may pose a risk for consumers when microbial pathogens are able to survive by the end of the production process or when cross-contamination occurs during post-processing operations (*i.e.*, cutting, slicing, packaging) (Christieans, Picgirard, Para, Lebert, & Gregori, 2018; Ganan, Hierro, Hospital, Barroso, & Fernández, 2013; Gómez et al., 2015).

Listeria monocytogenes is well known to be a real concern in the meat industry. Once introduced into meat processing plants through contaminated unprocessed raw materials, it can survive through the manufacturing process and adapt in processing plants, forming biofilms (De Candia, Morea, & Baruzzi, 2015; Gómez et al., 2015; Meloni et al., 2012). In all these environments, cross-contamination by *L. monocytogenes* can be a frequently occurring phenomenon (Pérez-Rodríguez et al., 2010; Possas, Carrasco, García-Gimeno, & Valero, 2017). The transfer ability of *L. monocytogenes* from equipment to dry-fermented sausages has been confirmed, as well as the occurrence of listeriosis outbreaks due to cross-contamination during slicing of this type of products (Anonymous, 2009; Lin et al., 2006; Vorst, Todd, & Ryser, 2006). According to the most recent EFSA report, 0.8 % of the ready-to-eat (RTE) fermented

sausages analysed at the processing stage did not comply with the microbiological criterion for *L. monocytogenes* (*i.e.* EC 2073/2005) showing levels higher than 100 CFU/g (EFSA, 2017).

L. monocytogenes proliferation is highly influenced by the nitrite addition in dry-fermented sausages (Hospital, Hierro, & Fernández, 2012; Perea-Sanz, Montero, Belloch, & Flores, 2018). Despite the important technological role of nitrite on the organoleptic characteristics and on the microbiological stability of dry-fermented sausages, the development of products with less nitrite is preferred by consumers and processors, as it is a precursor of nitrosamines, compounds with potential carcinogenic activity (Christieans et al., 2018; Hospital, Hierro, & Fernández, 2014). In this context, European authorities argue for the reduction of the maximum permitted concentration of nitrite in RTE meats (Hospital et al., 2017), which is currently 150 mg/kg in low salt foods, according to the European Directive 52/2006/EC. Furthermore, the Regulation (EC) 1333/2008 concerning the addition of nitrites and nitrates in traditional slow ripened sausages, such as chorizo, with maturation process of at least 30 days, provides the possibility of the exclusive application of nitrates up to 250 mg/kg as a curing salt (Perea-Sanz et al., 2018).

The application of high hydrostatic pressure (HHP) technology could help to limit the addition of nitrite in meat products, without affecting negatively their microbiological stability (Duranton, Guillou, Simonin, Chéret, & De Lamballerie, 2012; Fraqueza, Borges, & Patarata, 2018). The implementation of this non-thermal pasteurization technology has been proposed as an alternative to thermal processing to increase the safety and extend the shelf-life of dry-fermented sausages, since heat treatments may lead to unacceptable detrimental effects on their quality (Ducic et al., 2016; Matser, Krebbers, Van Den Berg, & Bartels, 2004). To optimize the application of HHP and to evaluate the factors that influence on its efficacy, mathematical models describing the *L. monocytogenes* inactivation induced by high-pressures as functions of technological parameters and intrinsic characteristics of RTE products have been developed (Possas, Pérez-Rodríguez, Valero, & García-Gimeno, 2017; Valdramidis, Patterson, & Linton, 2015).

Predictive models describing microbial behaviour in foods could be integrated into exposure assessment models, which may be used in Quantitative Microbial Risk Assessments (QMRA). By constructing a quantitative exposure assessment model simulating the transmission of *L. monocytogenes* along the chorizo production and distribution chain, the impact of HHP treatments on lowering microbial levels, as well as on changes in chorizo formulation, including lowering nitrite concentrations, can be assessed, assisting policy makers to come to decisions for increasing food safety. To date, no exposure assessment models for *L. monocytogenes* in dry-fermented sausages from raw material reception up to the consumption has been constructed.

The objectives of this study were: i) to build a probabilistic model to predict the fate of *L. monocytogenes* in Spanish chorizo sausage from mixing of raw materials up to consumption. ii) to evaluate the application of HHP technology as a measure to lower *L. monocytogenes* levels on the sliced-vacuum packed product. iii) to evaluate the impact of changes in formulation (i.e. nitrite reduction) in parallel with the application of HHP technology in chorizo safety regarding the presence of *L. monocytogenes*.

6.3. Material and Methods

6.3.1. Quantitative exposure assessment model overview

The probabilistic model includes nine steps from mixing of raw materials up to consumption and it was developed on the flow diagram shown in Figure 6.1. Various scenarios were evaluated reflecting chorizo sausages production chain in Spain while certain assumptions were made to develop the exposure assessment model. The model is developed in such a way that it can also be used as a practical tool for evaluating the survival of *L. monocytogenes* in sliced vacuum-packed chorizo, including the application of HHP to increase food safety. Each step of the model is described below. The steps are subdivided into two main sections: manufacturing process at industrial environments and distribution chain (Figure 6.1). A detailed overview of the model input variables is shown in Table 6.1.

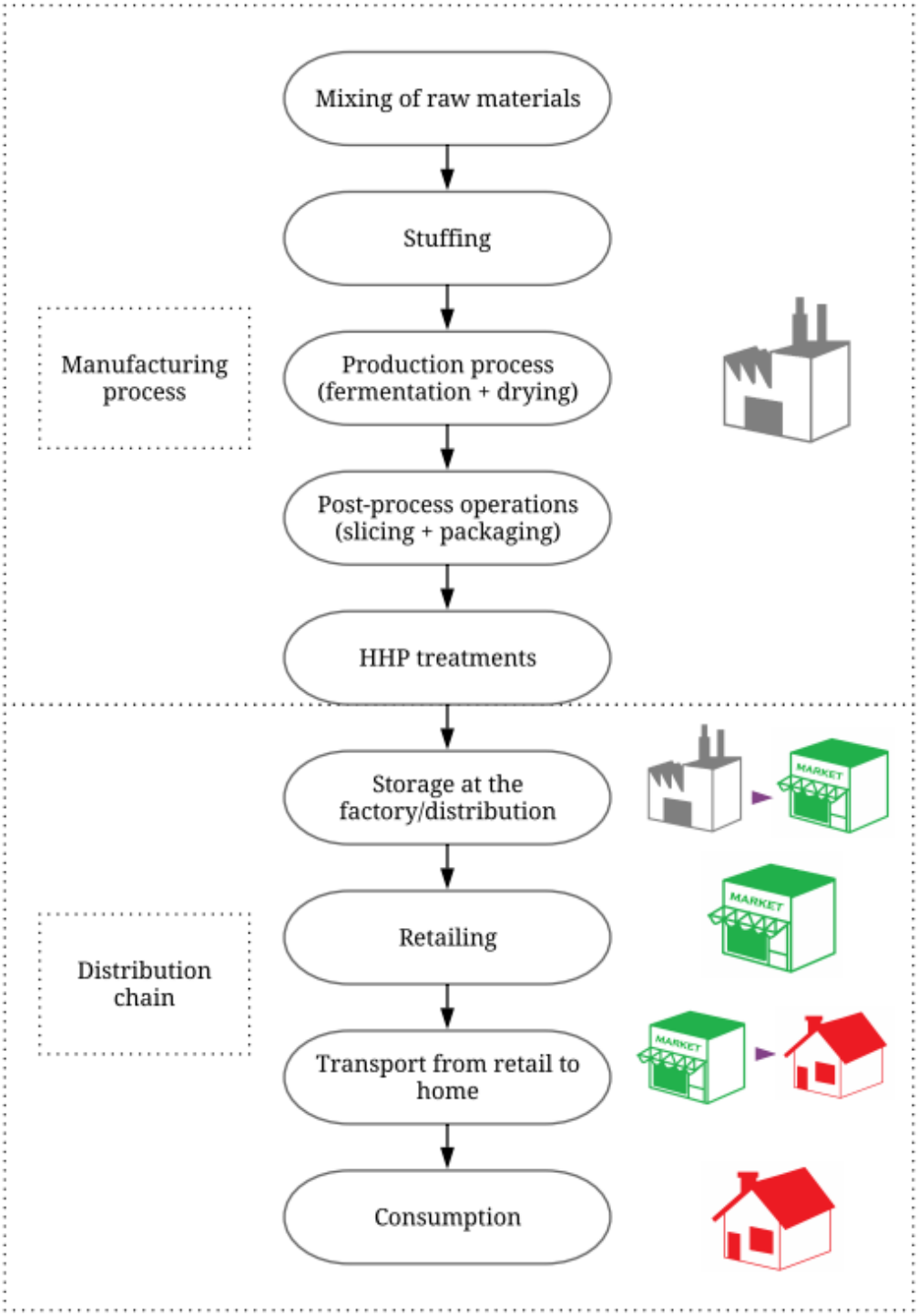

Figure 6.1- Flowchart of sliced vacuum-packed chorizo manufacturing process and distribution chain

Table 6.1- Detailed overview of the probabilistic model input variables

Model phase	*Input variable*	*Description*	*Distribution/model/value*	*Unit*	*References*
Mixing of raw materials	$[N_{PM}]$	*L. monocytogenes* concentration in pork meat batters	Normal(-1.43,0.16)	log cfu/g	Martín et al. (2011); Mataragas et al. (2015)
	%PM	percentage of pork meat in the mixture	Uniform(65,80)	%	Ordoñez & de la Hoz (2007)
	W_{batch}	weight of the mixture	1000	kg	-
Stuffing	$W_{sausage}$	weight of a sausage	800	g	-
Production (fermentation + drying)	t_{PP}	duration of production process	Uniform(20,54)	d	
	F	final fat	Normal(29.52,2.37)	%	NP
	$NaNO_2$	added nitrite	0-150	ppm	-
	$a_w(t_{PP})$	water activity of the product at the end of the production	Triangular(0.75,0.91,0.93)	-	NP
	$WPS(t_{PP})$	water phase salt	-125.02*a_w(t_{PP})^2+149.49*a_w(t_{PP})-21.56	%	Gunvig et al. (2016)
	pH_{48}	pH after fermentation	Triangular(4.76,4.87,5.40)	-	NP
	$pH(t_{PP})$	pH at the end of production process	Triangular(4.76,4.87,5.40)	-	NP
	$WL(t_{PP})$	water loss at the end of production	Normal(51.79, 0.70)	%	NP
	WL_d	water loss per day of production	$WL(t_{PP})/t_{PP}$	%	-
Post-process operations (slicing + packaging)	W_{slice}	weight of a slice	5	g	-
	W_{pack}	weight of a package of sliced sausages	80	g	-
	Tr	transfer coefficient from slicer blade to slices	Normal(0.71, 0.25)	%	Vorst et al. (2006)
	p_t	probability of *L. monocytogenes* presence in the slicing machine	Discrete(0:1;0.9143:0.0857)	-	Martín et al. (2011)
	N_{slicer}	initial level of contamination on the slicer region that contacts the slice per event	Uniform(1,1000)	cfu/slicer	-

Model phase	*Input variable*	*Description*	*Distribution/model/value*	*Unit*	*References*
HHP treatments	t_{HHP}	pressure holding-time	0-12	min	-
	P	pressure applied	400-600	MPa	-
	$a_w(t_{PP})$	water activity of the product	Triangular(0.75,0.91,0.93)	-	NP
Storage at the factory/distribution	t_{St}	storage at the factory duration	Uniform(0,36)	h	-
	T_{St}	storage temperatures at the factory	5	°C	Nauta et al. (2003)
Retail	*UBD*	use-by-date of the ready-to-eat products (primary shelf-life)	90	d	Marcos et al. (2013), Dalzini et al. (2015), Porto-Fett et al. (2008), Gounadaki et al. (2007)
	SSL	percentage of primary shelf-life equivalent to the maximum time products stay at retailing shelves (secondary shelf-life)	90	%	-
	t_{Rmin}	minimum storage time at retailing	Uniform(2,6)	h	-
	t_{Rmax}	maximum storage time at retailing	UBD*SSL	d	-
	t_R	storage time at retailing	Exponential(99%,$t_{Rmax,}$"Loc",t_{Rmin})	d	-
	T_R	temperature at retailing	Normal(3.70,1.78)	°C	Frisbee project data
Transport from retail to home	t_{Tr}	transport to home time	Uniform(0.25,2)	h	NP
	T_{Tr}	transport to home temperatures	Pert(4,10,25)	°C	Nauta et al. (2003)
Consumption	*PD*	date of purchase	$t_{St}+t_R+t_{Tr}$	d	Nauta et al. (2003)
	t_H	household storage time	Exponential((UBD-PD)/3)	d	Nauta et al. (2003)
	T_H	household temperatures	Normal(6.62,2.56)	°C	Carrasco et al. (2007)

*NP = Data not published obtained experimentally.

6.3.2. Manufacturing process at industrial environments steps

6.3.2.1. Mixing of raw materials

The sausages considered in this study are manufactured using a traditional technology and according to a traditional formulation, which consists of 65-80 % pork meat and 20-35 % pork back fat (Ordónez & de la Hoz, 2007). Lean pork meat and pork back fat are minced and subsequently mixed in a vacuum mixer with the addition of the following common ingredients per kilogram of meat mixture: 20 g sodium chloride, 20 g paprika, 10 g dextrose, 1.5 g garlic, 1.0 g oregano, 1.0 g black pepper and 1.0 g polyphosphates (Rubio et al., 2018). Finally, nitrite ($NaNO_2$) is added to the mixture at 150 ppm. This nitrite concentration is commonly added in chorizo formulations (Dalzini et al., 2014, 2015; Stollewerk, Jofré, Comaposada, Arnau, & Garriga, 2012; Stollewerk et al., 2011) and corresponds to the maximum concentration permitted in low salt foods (European Commission, 2006). The effects of reducing nitrite in chorizo formulation on the final levels of *L. monocytogenes* will be also evaluated (refer to section 6.4.4).

The first model step was built considering that pork meat is the main source of *L. monocytogenes* that enters the production chain. This assumption is reasonable since pork meat represents more than 60 % of the mixture and the levels of contamination from the other raw materials, mainly fat and spices, can be neglected. To obtain comparable data among different simulations, batch size (W_{batch}) was fixed at 1000 kg.

Bayesian analysis was applied to determine the distribution of *L. monocytogenes* concentration on the pork meat batter (Table 6.1) (Mataragas, Alessandria, Rantsiou, & Cocolin, 2015; Vose, 2008) based on data published by Martin et al. (2011). These authors determined the prevalence of *L. monocytogenes* from presence-absence data obtained at 10 small-scale factories in Spain. From the analysed samples, 47.4 % were tested positive.

6.3.2.2. Stuffing

At this step, the mixture composed by the raw ingredients is stuffed into natural casings (62-65 mm ø) to obtain the sausages. In this process, a large unit is split up into several smaller units, characterizing

a partitioning process. The diameter and weight of sausages can vary, but for the sake of simplicity the weight of each sausage ($W_{sausage}$) was fixed at 800 g. Thus, the number of sausages produced from one batch is $W_{batch}/W_{sausage}$= 1250 units (neglecting losses). The contamination level of the 800-g sausages was deduced from the level of contamination in the batch mixture, neglecting a potential growth of pathogens during stuffing. A homogeneous repartition of *L. monocytogenes* cells present in the initial batch is assumed, which is reasonable since the meat was minced before mixing (Lerasle et al., 2014). The number of cells in one sausage unit (N_{su}) can be calculated by a Poisson distribution (Equation 6.1), which is suitable to describe random patterns (Pérez-Rodríguez et al., 2014).

$$N_{su} = Poisson\,(N_{mix} \cdot \frac{W_{sausage}}{W_{batch}}) \qquad \text{Eq. (6.1)}$$

where N_{mix} is the total number of *L. monocytogenes* cells on the mixture.

6.3.2.3. Production process: fermentation + drying

After stuffing, the sausages are fermented for 48 h at 22-24°C/90 % RH and dried in a drying chamber at 14-18°C/75-90% RH from 3 to 6 weeks (Ockerman & Basu, 2015; Rubio et al., 2018). The model developed by Gunvig et al. (2016) available in the online software tool ConFerm (http://dmripredict.dk) was used to describe changes in *L. monocytogenes* levels along the production process (fermentation + drying) of chorizo sausages as a function of eight variables: duration of production process, final fat content, nitrite concentration, water phase salt concentration, pH at the end of the process, pH at 48 h after the beginning of the production process (end of fermentation), water loss percentage at the end of the production process and water loss per day of production (Table 6.1). This survival model was previously validated to data obtained in our lab during the production of chorizo sausages (unpublished results). Since chorizo is usually manufactured without selected starter cultures (Ortiz, López, Garriga, & Martínez-Suárez, 2014), the production process under study is not starter-assisted, thus the pH reduction during drying is not remarkable and the distribution of pH at 48

h (after fermentation) was assumed to be equal to the distribution of pH at the end of the production process (Table 6.1).

As not all the sausages will have the same number of *L. monocytogenes* cells after the production process, the number of cells in a sausage unit (N_{PP}) is deduced from a Poisson distribution (Equation 6.2).

$$N_{PP} = Poisson(10^{[N_{PP}]} \cdot W_{sausage}) \quad \text{Eq. (6.2)}$$

where $[N_{PP}]$ is the concentration of *L. monocytogenes* in the sausage unit in log cfu/g calculated by using the survival model developed by Gunvig et al. (2016).

6.3.2.4. Post-process operations: slicing and packaging

At this phase, the whole sausages are subjected to the slicing operation, which characterizes another partitioning process, as large units are subdivided into small ones. If the weight of a slice unit is W_{slice} = 5 g and cells are randomly distributed over the sausages, the number of cells that survives by the end of ripening process (i.e., coming from raw pork meat) in each slice (N_{part}) can be calculated by using Equation 6.3 (Membré & Boué, 2017).

$$N_{part} = Poisson\ (N_{PP} \cdot \frac{W_{slice}}{W_{sausage}}) \quad \text{Eq. (6.3)}$$

Besides the contamination from raw pork, we assumed that cross-contamination from equipment to slices could occur during the slicing operation of chorizo sausages.

The distribution of *L. monocytogenes* concentration on slices originated from the slicer ($N_{slicing}$) was estimated by Equation 6.4.

$$N_{slicing} = Binomial\ (N_{slicer}, p_t \cdot\ Tr) \quad \text{Eq. (6.4)}$$

where N_{slicer} is the number of cells present in the region of the slicer blade that contacts the sausages (donor surface); Tr is the transfer coefficient of cells from the slicer blade to the slices; and p_t is the probability of presence of *L. monocytogenes* cells on the slicer.

A schematic representation of cross-contamination during slicing is presented in Figure 6.2. The initial level of contamination on the region of the slicer blade that contacts the sausages (N_{slicer}) was assumed to follow a uniform distribution with values ranging from 1-1000 cfu, according to expert opinion (data not shown). The occurrence of a transfer event during simulation was based on a discrete distribution, defined by the probability of *L. monocytogenes* presence on the slicer machine (Table 6.1), that returns the value 0 when the slicer is not contaminated and 1 when the slicer is contaminated. The probability was derived from prevalence data of *L. monocytogenes* in slicing machines published by Borovic et al. (2014). These authors found out 8.57 % positive slicing machines out of 35 tested for the presence of the pathogen in food processing environments.

L. monocytogenes transfer coefficient values from the slicer to the product were estimated based on transfer data (*i.e.* slices concentration *versus* slice number) published by Vorst et al. (2006). For the extraction of transfer data from the graphs available in the published study, the DigitizeIt software version 2.2 (I. Bormann, Germany) was used. Transfer data obtained at the lower initial level of contamination on the slicer blade, *i.e.* 3 log cfu/blade, were used, since it is a scenario of contamination that could occur in reality (Vorst et al., 2006). The transfer coefficients were calculated according to Equation 6.5 (Pérez-Rodríguez et al., 2007). A probability distribution was fitted to the transfer coefficients values by using the @Risk software (Palisade, USA) (Table 6.1).

$$Tr\ (\%) = \log\left[\frac{cfu/slice}{cfu/blade} \cdot 100\right] \qquad \text{Eq. (6.5)}$$

where *Tr* (%) is the transfer coefficient; cfu/slice is the *L. monocytogenes* load in each slice; and cfu/blade is the initial pathogen concentration on the slicer blade.

Thus, the total number of *L. monocytogenes* cells present in each slice unit just after slicing is $N_{slice} = N_{part} + N_{slicing}$. After slicing, slices are vacuum-packed in plastic bags (polyamide/polyethylene) and vacuum sealed using a packer. In the modelling process, it was assumed that a cross-contamination event during slicing affected all slices contained in a pack, which corresponded to 16 slices. The total weight per pack was 80 g (W_{pack}). Contamination during packaging was assumed to be negligible.

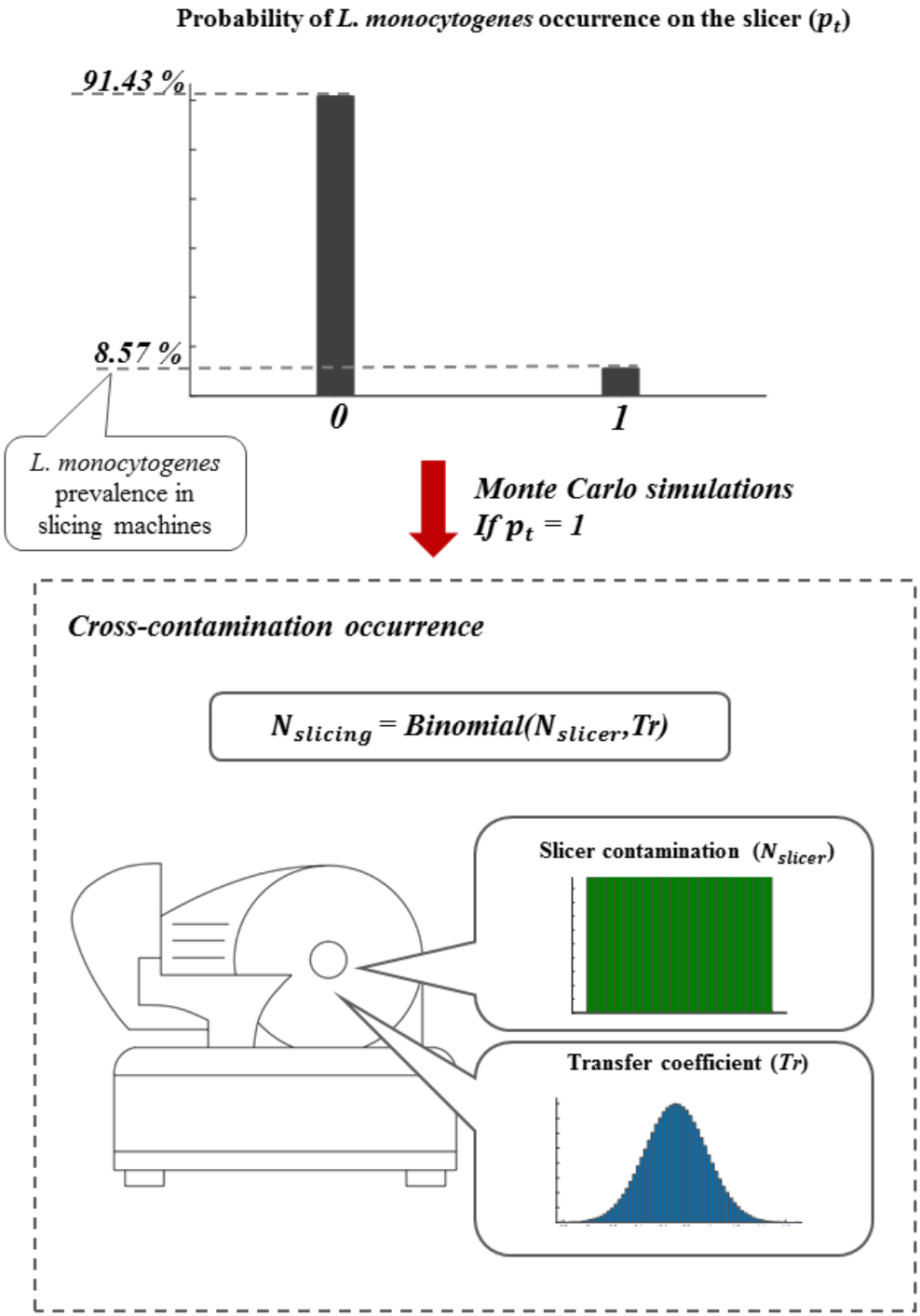

Figure 6.2- Scheme of the scenario of cross-contamination simulated in the current study: determination of the number of *Listeria monocytogenes* cells transferred during slicing ($N_{slicing}$)

6.3.2.5. High hydrostatic pressure treatments

The packs of sliced chorizo are subjected to HHP treatments at 400-600 MPa for 0-12 minutes in an industrial hydrostatic pressure unit using additive-free water as the pressure transmitting fluid. Pressure levels in the range of 400-600 MPa with short processing times of 3-7 min have been applied for the pasteurization of meat and meat products, but pressure-holding times as high as 12 min had also been evaluated for the inactivation of *L. monocytogenes* in dry-fermented products (Bajovic, Bolumar, & Heinz, 2012; Porto-Fett et al., 2010).

In all cases, the initial water temperature is set to 18°C, and *L. monocytogenes* inactivation during compression and decompression can be neglected (Rubio et al., 2018). To estimate the concentration of *L. monocytogenes* in the packs after HHP treatments the model developed by Rubio et al. (2018) in Spanish chorizo sausage, describing the pressure-induced inactivation of *L. monocytogenes* as a function of its a_w, pressure intensities and pressure-holding time was applied. The application of this model is appropriate, since in a previous study conducted in our laboratory (Possas et al., 2018), the pH and nitrite concentration did not affect significantly the inactivation levels of *L. monocytogenes* by HHP in a simulated meat medium, while the a_w, pressure and pressure-holding time were significant factors influencing on process lethality.

6.3.3. Distribution chain steps

The distribution chain encompasses the steps from storage at the factory up to consumption. To estimate the concentration of *L. monocytogenes* in sliced vacuum-packed chorizo products during the distribution chain, a survival mathematical model was developed based on survival data obtained in vacuum-packed sliced salami at different temperatures (5-25°C). *L. monocytogenes* concentration data at different times (*i.e.* log cfu/g *versus* storage time in days) were extracted from the survival curves published by Gounadaki et al. (2007) by using the software DigitizeIt software version 2.2.

The biphasic model (Equation 6.6; Cerf, 1977) was adjusted to survival data obtained at different temperatures and model parameters (k_{max1}, k_{max2} and f) were estimated for each temperature by using

the Matlab software 2017a (The Mathworks inc.). The relationship between temperature and biphasic model parameters was well described by a linear function (secondary modelling). Secondary model coefficients and goodness-of-fit indexes are presented in Table 6.2. By combining primary and secondary models, the number of survival cells can be estimated as a function of time and temperature. The goodness-of-fit of the resultant survival model is R^2_{adj} = 0.98 and *RMSE* = 0.1025.

$$\log(N) = \log(N_0) + \log(f \cdot \exp(-k_{max1} \cdot t) + (1 - f) \cdot \exp(-k_{max2} \cdot t)) \qquad \text{Eq. (6.6)}$$

N is the *L. monocytogenes* number at the end of each step in which the model is applied; N_0 is the number of cells in the packs at the beginning of each step; and k_{max1}, k_{max2} and *f* are biphasic model parameters.

Table 6.2- Coefficients and goodness-of-fit indexes of the secondary models describing the influence of storage temperature (*T*= 5-25 °C) on *L. monocytogenes* survival parameters in sliced vacuum-packed salami

Parameter	***Coefficients***[a]		***Goodness-of-fit indexes***	
	B_0	B_1	R^2_{adj}	***RMSE***
K_{max1}	0.2715 ± 0.1001	0.0442 ± 0.0060	0.9640	0.0488
K_{max2}	-0.0269 ± 0.0169	0.0065 ± 0.0010	0.9548	0.0081
f	0.9979 ± 0.0086	-0.0038 ± 0.0005	0.9653	0.0041

[a] Estimates ± Standard errors are reported.
Models in the form: $f(T) = B_0 + B_1 * T$
Models derived from survival data published by Gounadaki et al. (2007)

The application of the developed survival model in the current study is supported by a validation study performed with data obtained with chorizo samples in our laboratory at two temperatures: 10 and 15 °C (A_f = 1.13 and B_f = 0.93). However, to simulate temperatures lower than 5 ºC, a conservative approach has been adopted by fixing the k_{max2} at zero based on the fact that a decrease on temperature from 25 to 5 °C led to the reduction of this parameter to zero. Furthermore, by decreasing the temperature at this range, the *f* values increased to values approximately equal to 1, indicating homogeneity in temperature resistance within the *L. monocytogenes* population (i.e., all cells being

inactivated at the same rate). Model predictions show that by lowering temperature, a decrease of K_{max1} is observed and this trend is also observed in other studies with fermented sausages (Rubio et al., 2006; Simpson et al., 2008).

6.3.3.1. Storage at the factory/distribution

Once submitted to high-pressure treatments, the sliced vacuum-packed chorizo products are stored at the factory and subsequently transported to the retailers. It was assumed that the products could be immediately delivered to retailing after HHP treatments or that the maximum time elapsed from the end of HHP treatments until distribution is 36 h. Furthermore, products are stored/transported under controlled temperature at 5°C until they reach the retailers.

6.3.3.2. Retail

When the chorizo packs leave the factory, the use-by date (UBD) is set at 90 days, in accordance with Spanish manufacturers and other studies that reported the shelf-life of sliced dry-fermented sausages (Dalzini et al., 2015; Gounadaki et al., 2007; Marcos, Aymerich, Garriga, & Arnau, 2013; Porto-fett et al., 2008). Recontamination of the sliced dry-fermented sausages during retailing is negligible since the products are vacuum packed. However, in some cases, HHP treatments could be insufficient to completely inactivate *L. monocytogenes* cells, so cells could be able to survive up to the end of shelf-life. To consider the effect of retailing conditions on *L. monocytogenes* survival, the temperatures at retail were implemented as a Normal distribution with mean 3.71°C and standard deviation 1.78°C, based on data of the Frisbee Project available in http//www.frisbee-project.eu (Gwanpua et al., 2015). For time at retail, an exponential distribution was used defined by a maximum corresponding to 90 % of the UBD set for the products and a minimum varying from 2-6 h (Table 6.1).

6.3.3.3. Transport from retail to home

It was assumed that the transport from retail to home can last a minimum of 15 min and a maximum of 2 h. The temperature during transport is largely unknown, but in general foods are not refrigerated during transport by the consumer and at this phase there is an increase in temperature which depends

on many uncertain and variable factors. A pert distribution was used to describe the temperatures during transport from retail to home with minimum 4°C, most likely 10°C and maximum 25°C, as proposed by expert opinion published by Nauta et al. (2003). Differences in product temperature history profiles at various places throughout each pack are an additional source of variability, which are assumed to be included in this distribution.

6.3.3.4. Consumption

At household environments, the variability in average refrigerator temperature was described by a normal distribution with mean = 6.62°C and standard deviation = 2.52°C, according to data reported by Carrasco et al. (2007) in domestic refrigerators (n = 30) in south of Spain. To describe the distribution of times that the products are kept in the refrigerators, we assume an exponential distribution as in the case of retail times. This distribution describes the waiting time between two events (Nauta et al., 2003). The underlying assumption supporting the exponential distribution is that consumer behaviour regarding the storage time is influenced by the UBD. If PD is the day of purchase, UBD-PD is the time between purchase and use-by-date, which is the storage time in the household environment until the UBD is reached (Table 6.1). Knowing that the 95% quantile of the exponential distribution lies at three times its mean, the exponential distribution which gives 5% probability of a storage time exceeding the UBD has a mean μ=(UBD-PD)/3. The exponential distribution then describes the storage time after PD. As PD is variable, μ is variable too. The resulting distribution of storage times in the consumer refrigerator is derived by Monte Carlo simulations.

The exposure assessment ends at the moment consumers take the product from her/his refrigerator.

6.3.4. Model simulation

The probabilistic model was implemented in the Excel add-in @Risk (version 7.5, Palisade, Newfield, NY) and it was run using the Monte Carlo simulation technique. For each model simulation, 10,000 iterations were generated. The model output at each step was the probability distribution of the *L. monocytogenes* concentration and its prevalence per sausage, slices or packs (Table 6.3).

Table 6.3- Detailed overview of the probabilistic model outputs

Model phase	*Output*	*Description*	*Distribution/model/value*	*Unit*
Mixing of raw materials	N_{mix}	total *L. monocytogenes* load in the mixture	$N_{mix} = [N_{PM}] \cdot \%PM \cdot W_{batch}$	cfu/mixture
	$[N_{mix}]$	concentration in the mixture (batch)	$[N_{mix}] = \log(N_{mix}/W_{batch})$	log cfu/g
	p_1	prevalence in the mixture	$p_1 = 1 - \Pr(N_{mix} = 0)$	% batch
Stuffing	N_{su}	load per sausage unit after stuffing	$N_{su} = Poisson(N_{mix} \cdot W_{sausage}/W_{batch})$	cfu/sausage
	$[N_{su}]$	concentration in sausages after stuffing	$[N_{su}] = \log(N_{su}/W_{sausage})$	log cfu/g
	p_2	percentage of sausage units contaminated	$p_2 = 1 - \Pr(N_{su} = 0)$	% sausages
Production (fermentation + drying)	$[N_{PP}]$	concentration after the production process	$[N_{PP}] = [N_{su}] - \Delta\log(t_{PP})^a$	log cfu/g
	N_{PP}	load per sausage unit at the end of the production process	$N_{PP} = Poisson(10^{[N_{pp}]} \cdot W_{sausage})$	cfu/sausage
	p_3	percentage of sausage units contaminated	$p_3 = 1 - \Pr(N_{PP} = 0)$	% sausages
Post-process operations (Slicing + packaging)	N_{part}	load per slice unit after partitioning	$N_{part} = Poisson(N_{PP} \cdot W_{slice}/W_{sausage})$	cfu/slice
	$[N_{part}]$	concentration in a slice unit after partitioning	$\left[N_{part}\right] = \log(N_{part}/W_{slice})$	log cfu/g
	$N_{slicing}$	load per slice unit after slicing	$N_{slicing} = Binomial(N_{slicer}, T_r \cdot p_t)$	% slices
	$[N_{slicing}]$	concentration in a slice unit after slicing	$\left[N_{slicing}\right] = \log(N_{slicing}/W_{slice})$	log cfu/g
	N_{slice}	load per slice after partitioning + slicing	$N_{slice} = N_{part} + N_{slicing}$	cfu/slice
	$[N_{slice}]$	concentration after slicing	$[N_{slice}] = \log(N_{slice}/W_{slice})$	log cfu/g
	p_4	percentage of slice units contaminated	$p_4 = 1 - \Pr(N_{slice} = 0)$	% slices
	N_{pack}	load per pack of sliced sausage	$N_{pack} = (N_{slice} \cdot W_{pack}/W_{slice})$	cfu/pack
	$[N_{pack}]$	concentration per pack of sliced sausage	$\left[N_{pack}\right] = \log(N_{pack}/W_{pack})$	log cfu/g
	p_5	percentage of packs contaminated	$p_5 = 1 - \Pr(N_{pack} = 0)$	% packs

Model phase	*Output*	*Description*	*Distribution/model/value*	*Unit*
HHP treatments	$[N_{HHP}]$	concentration after HHP processing	$[N_{HHP}] = log([N_{HHP}]/[N_{pack}])^{b} + [N_{pack}]$	log cfu/g
	$N_{pack/HHP}$	load per pack after HHP processing	$N_{pack/HHP} = Poisson(10^{[N_{HHP}]} \cdot W_{pack})$	cfu/pack
	p_6	percentage of packs contaminated	$p_6 = 1 - \Pr(N_{pack/HPP} = 0)$	% packs
Storage at the factory/distribution	$[N_D]$	number of survivors after distribution	$[N_D] = [N_{HHP}] + \log(f \cdot \exp(-k_{max1} \cdot t_{St}) + (1-f) \cdot \exp(-k_{max2} \cdot t_{St}))^{c}$	log cfu/g
	N_D	load per pack after transport from factory to retail	$N_D = Poisson(10^{[N_D]} \cdot W_{pack})$	cfu/pack
	p_7	percentage of contaminated packs after transport from factory to retail	$p_7 = 1 - \Pr(N_D = 0)$	% packs
Retail	$[N_R]$	number of survivors after retailing	$[N_R] = [N_D] + \log(f \cdot \exp(-k_{max1} \cdot t_R) + (1-f) \cdot \exp(-k_{max2} \cdot t_R))^{c}$	log cfu/g
	N_R	load per pack after retailing	$N_R = Poisson(10^{[N_R]} \cdot W_{pack})$	cfu/pack
	p_8	percentage of contaminated packs after retailing	$p_8 = 1 - \Pr(N_R = 0)$	% packs
Transport from retail to home	$[N_{Tr}]$	number of survivors after transport from retail to home	$[N_{Tr}] = [N_R] + \log(f \cdot \exp(-k_{max1} \cdot t_{Tr}) + (1-f) \cdot \exp(-k_{max2} \cdot t_{Tr}))^{c}$	log cfu/g
	N_{Tr}	load per pack after transport from retail to home	$N_{Tr} = Poisson(10^{[N_{Tr}]} \cdot W_{pack})$	cfu/pack
	p_9	percentage of packs contaminated after transport from retail to home	$p_9 = 1 - \Pr(N_{Tr} = 0)$	% packs
Consumption	$[N_F]$	number of survivors at the moment of consumption	$[N_F] = [N_{Tr}] + \log(f \cdot \exp(-k_{max1} \cdot t_H) + (1-f) \cdot \exp(-k_{max2} \cdot t_H))^{c}$	log cfu/g
	N_F	load per pack at the moment of consumption	$N_F = Poisson(10^{[N_F]} \cdot W_{pack})$	cfu/pack
	p_{10}	percentage of packs contaminated at the moment of consumption	$p_{10} = 1 - \Pr(N_F = 0)$	% packs

[a] $\Delta\log(t_{PP})$=changes in *L. monocytogenes* concentration estimated by using the survival model of Gunvig et al. (2016);
[b] $\log([N_{HHP}]/[N_{pack}])$=inactivation levels estimated by using the model developed by Rubio et al. (2018);
[c] f, k_{max1} and k_{max2} are estimated by applying the secondary models presented in Table 6.2.

Although the model starting point was a contaminated batch of meat batter, prevalence may change from a mixture batch to sausages for example if, by chance, one or more sausages originated from a contaminated batch do not contain *L. monocytogenes* cells (*i.e.* partitioning effect) or after an effective HHP treatment able to eliminate, completely, *L. monocytogenes* contamination on sausages. The prevalence (p) at the end of each phase of the probabilistic model was deduced from $p = 1 - \Pr(X = 0)$, where *X* is the quantity of *L. monocytogenes* cells (cfu) in the mixture, sausages, slices or packs (Vose, 2008).

6.4. Results and Discussion

6.4.1. Distribution of *Listeria monocytogenes* by the end of manufacturing at industrial environments steps

A probabilistic model of *L. monocytogenes* in chorizo sausages from raw material up to the consumption phase has been developed. In this approach, probability distributions of the pathogen concentration in a dry-fermented product during the manufacturing production process and distribution chain (Figure 1) were derived from predictive models, experimental data and literature information. The results presented in this section are representative of the real manufacturing processes of chorizo sausage in Spain. The output mean values resulted of model simulations and their descriptive statistics are shown in Table 6.4.

The *L. monocytogenes* contamination level of a 1000-kg batch mixture (prevalence 100 %) was estimated to be -1.48 log cfu/g ± 0.11, which corresponds to a mean of 33 cfu/kg of mixture. The low standard deviation (*i.e.* 0.11) used to describe the contamination distribution in meat batters is supported by the fact that cell distributions are expected to be homogenous due to the mixing process applied. After partitioning of the initial mixture batch into 800-g sausage units during stuffing, the prevalence of *L. monocytogenes* did not decrease, with 100 % of sausage units contaminated with the pathogen (load per sausage = 27 ± 9 cfu/sausage).

Table 6.4- Overview of the model outputs, prevalence and cumulative probability of *L. monocytogenes* contamination level (X) per phase during the manufacturing production process

Model phase	***Mean ± SD (log cfu/g)***	***99th percentile (log cfu/g)***	***P(X ≤ 1 cfu/g)***	***P(X ≤ 10 cfu/g)***	***Prevalence (%)[a]***	***Unit***
Mixing of raw materials	-1.49 ± 0.11	-1.16	100%	100%	100%	batch
Stuffing	-1.49 ± 0.14	-1.14	100%	100%	100%	sausage
Production process (fermentation + drying)	-4.05 ± 0.19	-3.61	100%	100%	7.43%	sausage
Post-process operations (slicing + packaging)	-2.97 ± 0.21	-1.72	93%	98%	8.41%	packs

[a] Prevalence is equal to ≥ 1 cell of *L. monocytogenes* per unit.

During fermentation and drying processes, namely chorizo production process (step 3, Figure 6.1), the prevalence of *L. monocytogenes* on the sausage units reduced from 100 % to 7.56 %. The pronounced drop in the prevalence and the low mean concentration (*i.e.* -4.05 ± 0.19 log cfu/g, Table 6.4) can be attributed, as indicated by the simulations, to the fact that not all sausages are contaminated with the pathogen by the end of ripening of sausages. The low prevalence on sausages impacts on the mean concentration (in log cfu/g) resulting in very low values for this statistic. At this phase, water activity reduction during drying is one of the main factors influencing pathogen behaviour in the production process, which is characterized by a survival trend (Encinas, Sanz, García-López, & Otero, 1999; Hospital et al., 2012). The rapid pH drop during fermentation when starter cultures are added for chorizo manufacturing also reduces the survival ability of *L. monocytogenes* and contributes to ensure the safety of the product (Garriga et al., 2005; Ortiz et al., 2014). Since the manufacturing process under study is not starter-assisted, the low pH of chorizo is a result of the fermentation by pork meat microbiota and the presence of paprika and cayenne pepper in formulation (Marcos, Aymerich, & Garriga, 2005).

L. monocytogenes concentration and prevalence can vary greatly between different studies and different types of fermented sausages. For instance, in the study by Gómez et al. (2015), who investigated the occurrence of *L. monocytogenes* in RTE meat products and meat processing environments in six Spanish provinces, 36.84 % out of 57 fermented sausages, including chorizo and *salchichón*, were contaminated with the pathogen. Furthermore, these authors quantified *L. monocytogenes* in 11 raw-cured samples just after the production process, which contained 10-910 cfu/g. In the study of Martín et al. (2011), 15.8 % of 19 *fuet* fermented sausages analysed at 10 small scale factories in Catalunya were positive for the pathogen, while 12 out of 192 (6.3 %) of dried pork sausages were contaminated with *L. monocytogenes* in Eastern Spain (Doménech, Jimenez-Belenguer, Amoros, Ferrus, & Escriche, 2015). Therefore, results from the probabilistic model developed in this study, although different, fell within the range of reported values for prevalence and concentration at the production stage. Furthermore, the model might be applied to estimate the prevalence and the

distribution of *L. monocytogenes* in fermented sausages under different scenarios of product formulation, by setting different input variable values.

The operation that follows the production process is the slicing of matured chorizo sausages. The number of cells present in a slice after slicing is dependent of two events: partitioning of cells that are present in the originating sausage unit and transfer of cells present in the slicing machine. Under the studied conditions, partitioning led to very low pathogen concentrations in slices (maximum 1 cell/slice). According to model simulation results, the prevalence of *L. monocytogenes* in slices increased from 0.04 % (only pork meat as contamination source) to 8.38 %, when cross-contamination occurred (pork meat and slicer as contamination sources). The distribution of *L. monocytogenes* cells transferred to slices is shown in Figure 6.3. According to our results, a relatively high level of the pathogen can be transferred from the slicing machines to slices (0.59 ± 0.48, with a maximum of 1.69 log cfu/g), which indicate that measures must be taken to avoid cross-contamination in dry-fermented sausages industries, with special attention to the cleaning and disinfection of the slicing machines.

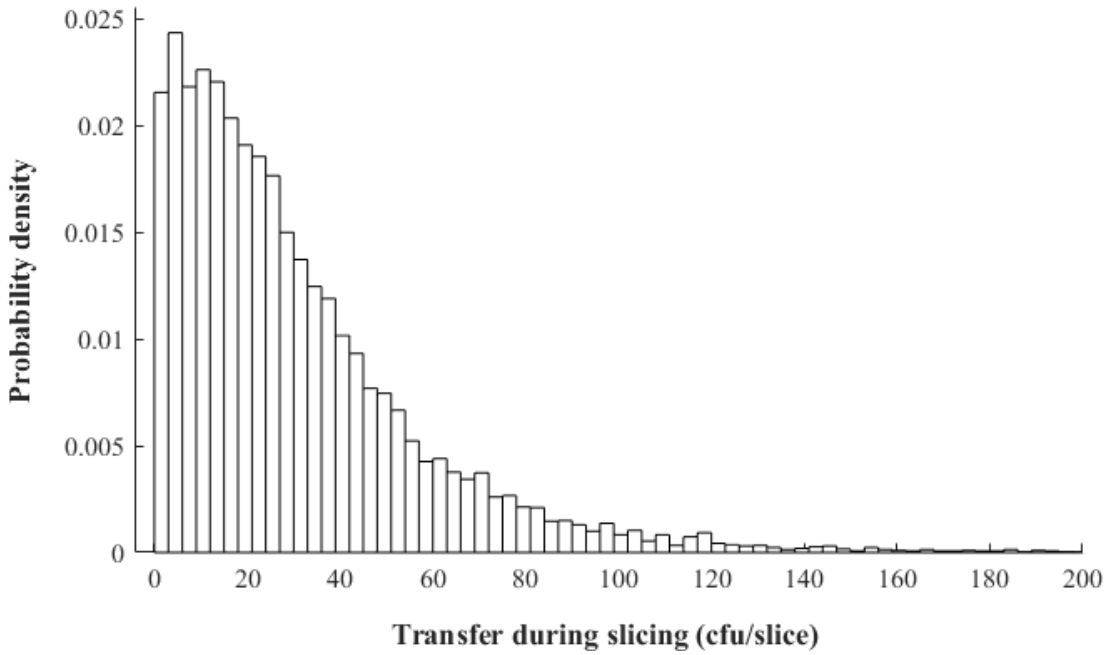

Figure 6.3- Simulated distribution of *Listeria monocytogenes* transferred to slices of chorizo during the slicing operation

Once chorizo sausages are sliced, products are vacuum-packed. Considering that a cross-contamination event influences a whole pack of product, the prevalence of the pathogen in sliced-vacuum packed chorizo by the end of post-process operations step was also 8.38 % with a mean concentration per pack of 0.59 ± 0.48 cfu/g. Vacuum packaging is applied to prevent the growth of aerobic microorganisms to avoid/retard foods deterioration (Ahn & Byungrok, 2007). However, vacuum packaging also favours the survival of facultative or anaerobic microorganisms such as *L. monocytogenes* by inhibiting competitive microorganisms and preventing the further reduction of a_w that slices would suffer during storage (Jofré, Aymerich, & Garriga, 2009). As a consequence, vacuum packaging of sliced salami resulted in the slower destruction of *L. monocytogenes* in comparison with aerobic packaging (Gounadaki et al., 2007).

Results confirm that once introduced into meat processing plants, *L. monocytogenes* can overcome chorizo manufacturing process. Even if cells are present in low levels in sliced-vacuum packed products, inadequate storage temperatures during the distribution chain could enable the growth or recovery of cells from sub-lethal injuries. This can be extended to dry-fermented products that are not submitted to slicing and are sold as whole sausages. For instance, in the study by Gómez et al. (2015), one sample of raw-cured sausage was contaminated with 190 cfu/g at half shelf-life of the product, a value that exceeds the food safety limit concerning *L. monocytogenes.*

In general, different measures can be applied to control *L. monocytogenes* levels in RTE dry-fermented meat products at industrial environments, from raw materials reception until final products packaging. Regarding raw materials, the certification of suppliers, especially meat providers, would assist in reducing the initial level of contamination present in batch mixtures (Mataragas et al., 2015). Food-contact surfaces and equipment, mainly slicing machines should be designed specially to enable the performance of correct and periodic cleaning and disinfection procedures (Gómez et al., 2015). Furthermore, the efficacy of these cleaning and disinfection procedures must be confirmed periodically.

6.4.2. Effect of the initial concentration of *Listeria monocytogenes* in pork meat batter

In this study, the initial level of contamination in pork meat batter was derived from prevalence data obtained in meat processing environments as reported by one specific study (Martín et al., 2011) (Table 6.1). However, the mean pathogen concentration in the pork meat batter could vary, which would influence the prevalence and concentration of *L. monocytogenes* in final products. To determine the impact of higher initial contamination levels on *L. monocytogenes* prevalence in the product, after packaging and at the moment of consumption, a scenario analysis was performed by changing the mean initial concentration of *L. monocytogenes* (-1.43-3 log cfu/g) (Figure 6.4).

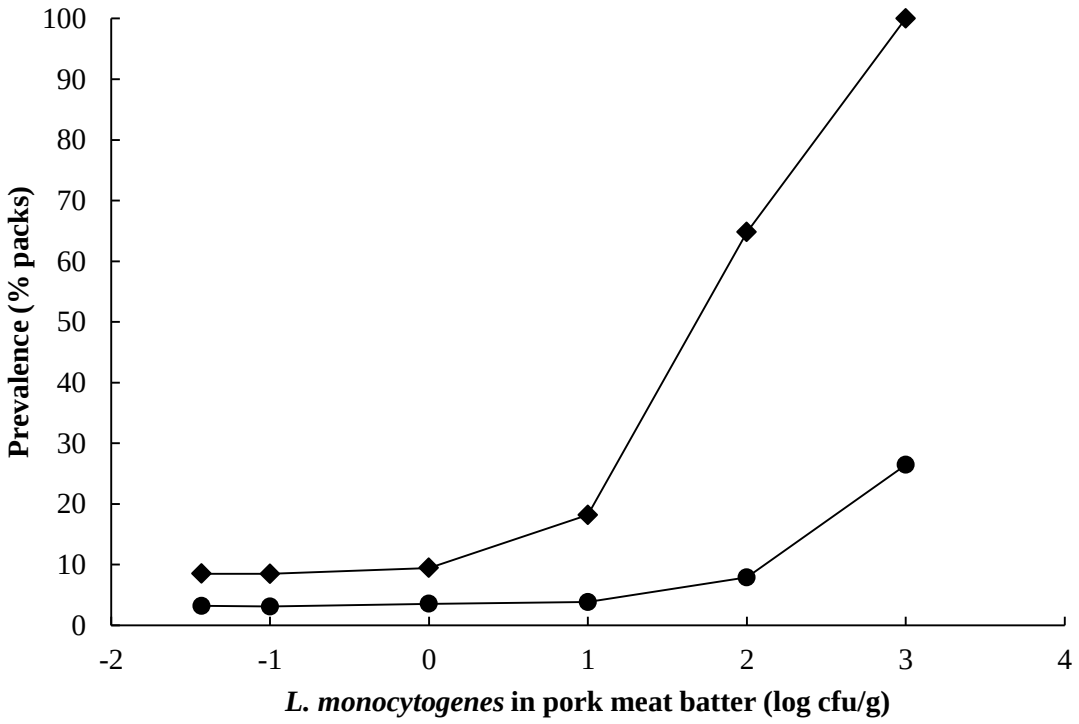

Figure 6.4- Influence of the initial level of contamination of the pork meat batter on the prevalence of *Listeria monocytogenes* in sliced vacuum-packed chorizo. (♦) by the end of packaging operation; (●) at the consumption phase

The increase from -1.43 to 0 log cfu/g did not influence the prevalence of *L. monocytogenes* in chorizo packs by the end of packaging. The highest impact was observed when the mean concentration was

equal to 3 log cfu/g resulting in 100 % of prevalent packs. In this extreme scenario, the 99th percentile of the distribution of *L. monocytogenes* in final products was 1.18 log cfu/g, which was 4 times higher than that obtained in the baseline model (Table 6.4). A similar behaviour was observed for products at the moment of consumption, although with lower prevalence values likely due to the effect of storage, highlighting the fact that an initial contamination of 1 log cfu/g did not lead to a perceptible rise of prevalence in contrast to what was obtained in products after packaging.

6.4.3. The fate of *Listeria monocytogenes* in sliced-vacuum packed chorizo during the distribution chain: impact of HHP treatments

The application of high-pressures is proposed as an intervention measure to control *L. monocytogenes* in the RTE meat products under study. In this section the effects of various HHP treatments on the final levels of the pathogen on sliced vacuum-packed chorizo were evaluated. This is the first attempt to quantitatively assess the application of HHP on the final levels of *L. monocytogenes* in an RTE dry-fermented product. Other authors had also quantitatively assessed the application of HHP application to reduce its levels in a ready-to-cook poultry meat (Lerasle et al., 2014).

The level of contamination at the consumption phase when no pressurization is applied was estimated to be low, with 99th percentile being -1.31 log cfu/g, a maximum of 0.13 log cfu/g and prevalence equal to 3.0 % of contaminated packs. In the management approach developed by Mataragas et al. (2015), in which stochastic modelling and meta-analysis were applied to estimate the risk of *L. monocytogenes* survival in sliced vacuum-packed dry-fermented products, the percentage of non-conforming products at the time of consumption, *i.e.*, the fraction with levels above 2 log cfu/g, was estimated to be 0.202%. In contrast, under the conditions evaluated in the current study, no packs were predicted to contain more than 2 log cfu/g of *L. monocytogenes* at the time of consumption. Nevertheless, in countries such as the United States, in which a zero tolerance is applied for the presence of the pathogen, the product under study would not be considered acceptable for importation.

Pressure-treatments exert a decontamination effect, reducing both microbial prevalence and levels in sliced vacuum-packed chorizos. As pressure-treatments at 400-600 MPa for 3-7 min are commonly

applied at the industrial level, the effects of treatments at 400-600 MPa for 5 min in *L. monocytogenes* concentration and prevalence are shown in Figure 6.5. Treatments at 400-500 MPa are equally effective in reducing the percentage of contaminated packs of chorizo. By increasing the pressure level to 600 MPa, lethality is considerably increased, with pathogen prevalence equal to 4.76 % just after pressurization and 0.11 % at the consumption phase. Thus, by applying a treatment at 600 MPa for 5 min, prevalence is approximately 45 % and 96 % lower in comparison with not pressurized packs, just after pressurization and at the consumption phase, respectively.

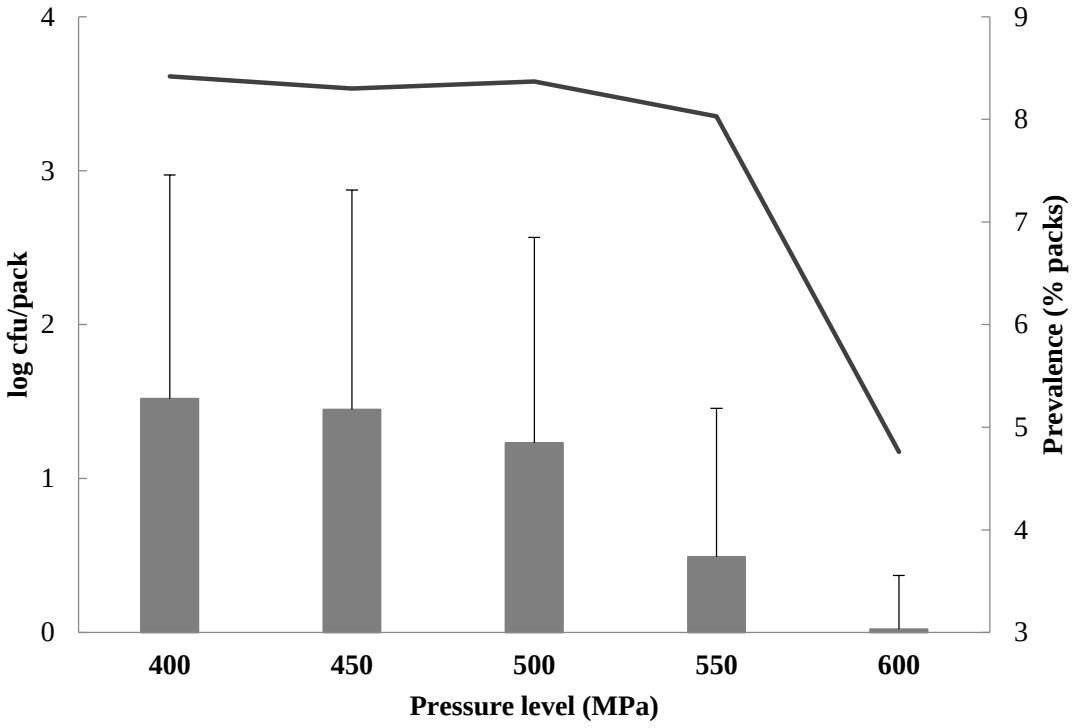

Figure 6.5- Effects of HHP treatments at different pressure intensities for 5 min in model outputs, just after pressurization: (■) mean concentration per pack; (⊣) 99th percentile of the distribution of *Listeria monocytogenes* concentration per pack; (—) percentage of contaminated packs

For pressure treatments at 600 MPa, the effects of increasing pressure-holding times from 0 to 12 min in *L. monocytogenes* prevalence in final products at all different steps of the distribution chain can be seen in Figure 6.6.

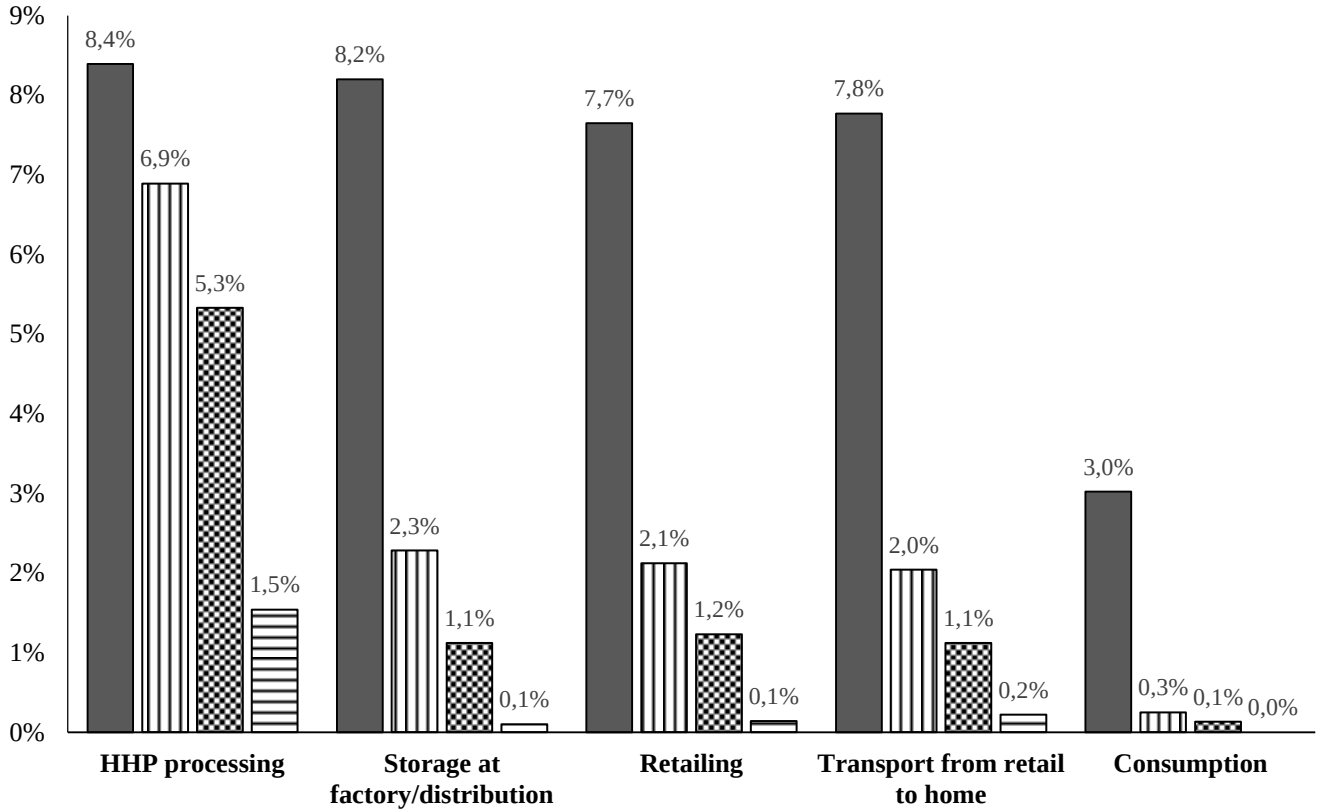

Figure 6.6- Impact of various HHP treatments on *Listeria monocytogenes* prevalence in sliced vacuum-packed chorizo products at the different steps of the logistic distribution chain: (■) without treatment; (▥) treatment at 600 MPa/ 3 min; (▩) treatment at 600 MPa/6 min; (▤) treatment at 600 MPa/9 min

During a pressure-treatment at 600 MPa/3 min, which is the most commercially applied based on operational costs, the prevalence of *L. monocytogenes* decreases from 8.41 (no pressure treatment) to 6.89 % in products just after pressure treatment (Figure 6.6). Moreover, when this pressure treatment is applied, it is estimated that 100 % of the 80-g chorizo packs contains ≤ 1 cfu/g at the time of consumption. Pressure-treatments at 600 MPa/10 min would be sufficient to reduce the prevalence of *L. monocytogenes* to 0 % of chorizo packs contaminated at the time of consumption. Thus, a treatment at 600 MPa/3 min could assure products' compliance with the regulation EC 2573/2001, while treatments at 600 MPa/10 min would lead to the absence of *L. monocytogenes* in sliced-vacuum packed chorizo at the time of consumption.

At the storage at the factory/distribution phase, a marked prevalence drop occurred in pressure-treated packs in comparison with non-pressurized packs (Figure 6.6). For instance, after applying a treatment at 600 MPa/6 min, prevalence in packs decreased from 5.33 % to 1.12 % by the end of storage at the factory/distribution phase. When pressurisation is not applied, prevalence in chorizo packs only decreased from 8.38 % (prevalence after packaging) to 8.20 % by the end of storage at the factory/distribution. In general, no significant reductions in prevalence occur from storage at the factory to transport-to-home phases, in both pressure and non-pressure treated packs. However, the prevalence did drop during household storage in both pressure-treated and non-treated packs, which is probably associated with the higher domestic storage temperatures in comparison with the storage temperatures at the previous steps of the distribution chain.

According to many studies the increase in storage temperatures increases the inactivation rate of *L. monocytogenes* in fermented sausages (Byelashov et al., 2009; Gounadaki et al., 2007; Lindqvist & Lindblad, 2009; Menéndez, Rendueles, Sanz, Capita, & García-Fernández, 2015; Simpson et al., 2008). Based on this positive relationship, some authors constructed a decision support tool with quantitative data of *L. monocytogenes* survival in vacuum-packaged fermented sausages during post-process storage that can be applied to predict the desired storage time-temperature combinations to achieve additional pathogen reductions before their distribution (Mataragas, Alessandria, Rantsiou, & Cocolin, 2015). As an additional storage before distribution would represent the reduction of shelf-life

of the products, the application of a post-lethality treatment such as HHP to inactivate *L. monocytogenes* seems to be more reasonable.

The results from this study show that HHP application as an a nonthermal pasteurization method can be a powerful intervention strategy for controlling *L. monocytogenes* in sliced-vacuum packaged chorizo as a part of a good overall hazard analysis critical control point (HACCP) program. Besides process lethality efficacy, another important aspect to be considered before applying this technology is the impact of pressures on the sensory and nutritional quality of foods. Investigation published so far indicated that the changes induced by HHP in dry-cured meat products in terms of acceptability are negligible (Campus, 2010; Hayman, Baxter, O'Riordan, & Stewart, 2004). In fact, the application of HHP treatments at 400 MPa during 2.5-16 min improved the sensory attributes of Portuguese chorizo, including the bright aspect of the whole sausage, firmness and cohesion (Alfaia et al., 2015). In agreement with Alfaia et al., (2015), no detrimental effects were detected on the sensory properties of *salchichón* and salami by applying pressure treatments at 500 MPa/5 min and 600 MPa/3 min, respectively (Gill & Ramaswami, 2008; Rubio, Martínez, García-Cachán, Rovira, & Jaime, 2007). The impacts of pressurization on the nutritional quality of RTE dry-fermented products must be further assessed.

6.4.4. Effect of nitrite reduction in parallel with HHP application on the final distribution of *Listeria monocytogenes*

The developed approach allows to evaluate the impact of nitrite reduction/removal concerning *L. monocytogenes* levels in the dry-fermented product formulation under study when an HHP treatment is applied.

Nitrite addition has a strong influence on pathogen reductions at the production process step (step 3, Figure 6.1), with the 99^{th} of the distribution of *L. monocytogenes* per sausage unit ranging from 1 to 15 cfu/sausage when nitrite is added at 150 ppm and without nitrite addition, respectively. Model simulations indicated that when nitrite is not added to chorizo formulation, the prevalence of *L. monocytogenes* by the end of drying is 97.15 % of contaminated sausage units, almost 90 % higher

than when 150 ppm nitrite is added. Furthermore, at this stage, reductions in *L. monocytogenes* levels vary from 0.8 to 2.6 log cfu/g when 0 and 150 ppm of nitrite is added, respectively. These reductions are in accordance with results of *Listeria* survival during ripening of Spanish *salchichón* formulated with different nitrite concentrations (Hospital et al., 2012).

Model simulations indicated that if nitrite was not present in formulation, packs of the product could carry more than 100 cfu/g by the end of post-processing operations in case the initial level of contamination in pork meat batter exceeded 2.6 log cfu/g. In the absence of nitrite, a *L. monocytogenes* level higher than 3.5 log cfu/g in pork meat batters could lead to non-compliant products at the time of consumption. In such cases, the application of HHP would be essential to guarantee compliance with current regulations.

The effects of reducing nitrite concentrations from chorizo formulation in the levels of *L. monocytogenes* at the moment of consumption are presented in Table 6.5. In general, at the consumption stage, there are no differences in *L. monocytogenes* prevalence in packs of chorizo with different nitrite concentrations, submitted to the same pressure-treatments (Table 6.5). For pressure-treatments at 600 MPa during 3-12 min, the probability of packs being contaminated with ≤ 1 cfu/g is 100 % at the time of consumption, which indicates that the chosen pressure-time combinations effectively reduce *L. monocytogenes* levels in chorizo, independent of the nitrite concentration present in formulation, under the studied conditions.

To guarantee pathogen absence in sliced-vacuum packed chorizo, the prevalence in final packs at consumption must be reduced to zero. This occurs when the packs are pressurized at 600 MPa for 12 min, independent of the added amount of nitrite (0-150 ppm). According to these results, the removal/reduction of nitrite from chorizo formulations is feasible from the microbiological point of view when pressure-treatments are applied as a post-process lethality treatment in the final products.

Table 6.5- Effects of reducing nitrite concentrations in parallel with the application of pressure-treatments at 600 MPa for different holding-times (0-12 min) on the distribution of *L. monocytogenes* in chorizo by the end of the distribution chain (*i.e.* consumption phase)

Nitrite (ppm)	Holding time (min)	Prevalence[a] (% packs)
150	0	3.04
	3	0.29
	6	0.09
	9	0.02
	12	0.00
100	0	3.17
	3	0.41
	6	0.08
	9	0.00
	12	0.00
50	0	3.05
	3	0.27
	6	0.11
	9	0.02
	12	0.00
0	0	3.07
	3	0.34
	6	0.15
	9	0.01
	12	0.00

[a] Prevalence is equal to ≥ 1 cell of *L. monocytogenes* per unit.

The probabilistic model enables to recommend combinations of HHP treatments and nitrite concentrations in chorizo formulation to guarantee an acceptable *L. monocytogenes* concentration at the time of consumption. The impacts of nitrite reduction in the organoleptic characteristics of dry-fermented products must be taken into consideration, due to its important technological role as a curing component. Alternatively, further studies of the microbiological and sensory impacts of nitrite substitution as curing salt in dry-fermented sausages in parallel with HHP technology application would be of great relevance.

6.5. Conclusions

This study illustrates how a probabilistic model of *L. monocytogenes* in dry-fermented sausages from raw materials up to the consumption phase can be constructed by linking currently available predictive models and available data. The quantitative exposure assessment performed showed that *L. monocytogenes* is able to survive the manufacturing production process and distribution chain of sliced vacuum-packed chorizo, especially when cross-contamination during post-process operations occurs. HHP is a powerful nonthermal pasteurization method for controlling *L. monocytogenes* in the final products, reducing pathogen concentration and prevalence. Based on the probabilistic model developed in this study, healthier products (i.e., nitrite-reduced) obeying EU/US regulations for *L. monocytogenes* could be developed considering the application of HHP as an intervention technology to increase their microbiological safety during shelf-life. Overall, the results of this study will assist food business operators to make decisions regarding reformulation and to ensure the safety of dry-fermented products.

6.6. References

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5. Conclusions

FIRST: According to the updated overview of microbial transfer phenomena in food processing, cross-contamination dynamics can be influenced simultaneously by a series of environmental and food matrix inherent factors and the development of mathematical models enables a better understanding of the individual contribution of each one of these factors. Based on these models, corrective measures to reduce cross-contamination issues in food processing environments may be applied (*Chapter I*).

SECOND: The use of compartmental mechanistic models could allow to better understand the influence of food processing factors and the indirect mechanisms involved in cross-contamination. However, the high variability and uncertainty sources during transfer phenomena represent a limitation of these models. The use of alternative performance indices for model evaluation, such as Acceptable Simulation Zone and Total Transfer Potential, can offer an added value to facilitate their application in food process operations (*Chapter I*).

THIRD: Based on a thorough review of inactivation models of *L. monocytogenes* in foods treated with High Hydrostatic Pressure (HPP) technology, it was demonstrated that the most commonly used models to describe the inactivation kinetics are the Weibull, log-logistic, Baranyi and Gompertz. Polynomial equations generated based on Response Surface Methodology are usually applied to study the influence of technological parameters, food composition, intrinsic factors and food additives on the pressure-induced inactivation of *L. monocytogenes* and to optimize the application of HHP at the industrial level. In addition, there are available, in the literature, logistic models of HHP-induced microbial inactivation, which are more realistic approaches as they consider the recovery of injured cells during storage of processed foods (*Chapter II*).

FOURTH: According to the review of existing scientific data, the pressure intensities and the pressure holding time were the most important technological factors governing HHP efficacy. Furthermore, controversies between studies regarding the significance of different factors, such

as fat and protein content, on pressure-lethally evidence the need for product-oriented approaches when evaluating HHP processing to inactivate bacteria (*Chapter II*).

FIFTH: According to results obtained from a simulated meat medium submitted to HHP, this technology was able to reduce *L. monocytogenes* contamination by 6.2 log cfu/g. The developed polynomial model identified pressure, time and NaCl as significant factors influencing HP-lethality while nitrite and pH were not statistically significant. Moreover, NaCl showed a strong interaction with pressure intensities on the *L. monocytogenes* inactivation, demonstrating that food components/additives and technological parameters can simultaneously influence on pressure-induced inactivation *(Chapter III)*.

SIXTH: Results generated from the application of HHP in Spanish chorizo sausage demonstrated that a_w, pressure and time significantly affected the inactivation of *L. monocytogenes*, although the reductions were lower than in the simulated meat medium (0-3.7 log cfu/g). Differences in inactivation levels between the modelling approaches can be attributed to the characteristics of the medium under study (*Chapter IV*).

SEVENTH: The study performed with the simulated meat medium proved that the development of models in culture media or food model systems leads to a better understanding of the influence of several factors on HHP lethality, enabling the optimization of the development of product-oriented modelling approaches, considering only the most significant factors affecting HHP technology effectiveness (*Chapter III and IV*).

EIGHTH: The polynomial models developed in the simulation meat medium and Spanish chorizo sausage were proven to be valid tools to determine the combinations of pressure and pressure holding-times required to meet a given target of *L. monocytogenes* inactivation as a function of the sodium chloride concentration or a_w of the meat products. In both models, a baroprotective effect on *L. monocytogene*s cells was evidenced by decreasing the a_w. The modelling approaches developed can help food manufacturers to optimise and manage HHP treatments in order to ensure Spanish chorizo sausage safety in accordance with the regulations

established for RTE meat products with respect to *L. monocytogenes* levels (*Chapters III and IV*).

NINETH: The application of the Quantitative Microbial Risk Assessment methodology through the development of a probabilistic Exposure Assessment model proved that *L. monocytogenes* is able to survive the manufacturing production process and distribution chain of sliced vacuum-packed chorizo, especially when cross-contamination during post-process operations occurs. In this context, it was proven that HHP is a good alternative for controlling *L. monocytogenes* in the final products, reducing pathogen concentration and prevalence and ensuring the compliance with EU/US regulations (*Chapter V and VI*).

TENTH: The microbiological safety of raw-cured sausages concerning *L. monocytogenes* is not compromised by the nitrite reduction/absence when appropriate HHP treatments are applied in packed products, which confirms that the application of this technology assists the development of healthier foods (*Chapter VI*).

ELEVENTH: Overall, outcomes from this thesis represent for a successful proof of application of predictive microbiology, demonstrating that mathematical models, when oriented to a specific product and processing technology, can be deployed as reliable and efficient tools to assist food business operators to make decisions regarding reformulation of raw-cured sausages and to ensure the safety of these products by means of HHP technology application.