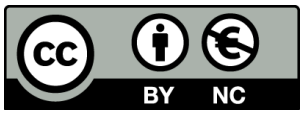
Universitat de Girona

COMBINED APPLICATION OF EXPERIMENTAL AND PREDICTIVE MODELLING APPROACHES TOWARDS THE MICROBIAL SAFETY OF READY-TO-EAT MEAT PRODUCTS

Cristina Serra Castelló



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IRTA

Institut de Recerca i Tecnologia Agroalimentàries

DOCTORAL THESIS

Combined application of experimental and predictive modelling approaches towards the microbial safety of ready-to-eat meat products

CRISTINA SERRA CASTELLÓ

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Doctoral thesis

COMBINED APPLICATION OF EXPERIMENTAL AND PREDICTIVE MODELLING APPROACHES TOWARDS THE MICROBIAL SAFETY OF READY-TO-EAT MEAT PRODUCTS

Cristina Serra Castelló

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WE DECLARE:

That this thesis, entitled "Combined application of experimental and predictive modelling approaches towards the microbial safety of ready-to-eat meat products", presented by CRISTINA SERRA CASTELLÓ to obtain a doctoral degree, has been carried out under our supervision and meets the requirements to opt for an International Doctorate.

For all intents and purposes, we hereby sign this document:

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List of Publications

The present PhD thesis has been written as a compendium of peer-reviewed Articles in accordance with the regulations of the University of Girona.

The PhD thesis is comprised of the following papers:

Peer-reviewed Articles[1]:

1) Serra-Castelló, C; Possas, A; Jofré, A; Garriga, M; Bover-Cid, S. (2022). *Enhanced high hydrostatic pressure lethality in acidulated raw pet food formulations was pathogen species and strain dependent*. Food Microbiology, 104, 104002. DOI: https://doi.org/10.1016/j.fm.2022.104002

   ISSN: 0740-0020, Impact Factor: 6.374, ranked 22/144 in the category of *Food Science & Technology* (1st quartile)[1].

2) Serra-Castelló, C; Possas, A; Jofré, A; Garriga, M; Bover-Cid, S. (2022). *High-pressure processing inactivation of Salmonella in raw pet food for dog is enhanced by acidulation with lactic acid.* Animal Feed Science and Technology, 290, 115347. DOI: https://doi.org/10.1016/j.anifeedsci.2022.115347

   ISSN: 0377-8401, Impact Factor: 3.313, ranked 11/62 in the category of *Agriculture, Dairy & Animal Science* (1st quartile)[1].

3) Serra-Castelló, C.; Possas, A; Jofré, A; Garriga, M; Bover-Cid, S. (2022). *High pressure processing to control Salmonella in raw pet food without compromising the freshness appearance: The impact of acidulation and frozen storage*. Food Microbiology, 109, 104139. DOI: https://doi.org/10.1016/j.fm.2022.104139

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4) Serra-Castelló, C; Jofré, A; Belletti, N; Garriga, M; Bover-Cid, S. (2021). *Modelling the piezo-protection effect exerted by lactate on the high pressure resistance of Listeria monocytogenes in cooked ham.* Food Research International, 140, 110003. DOI: https://doi.org/10.1016/j.foodres.2020.110003

   ISSN: 0963-9969, Impact Factor: 7.425, ranked 13/144 in the category of *Food Science & Technology* (1st quartile).

5) Serra-Castelló, C; Ferrocino, I; Jofré, A; Cocolin, L; Bover-Cid, S; Rantsiou, K. (2021). *Unravelling the molecular mechanisms underlying the protective effect of lactate on the high-pressure resistance of Listeria monocytogenes.* Biomolecules, 11(5), 677. DOI: https://doi.org/10.3390/biom11050677

   ISSN: 2218-273X, Impact Factor: 6.064, ranked 75/297 in the category of *Biochemistry & Molecular Biology* (2nd quartile).

[1] Publications are listed according to the order presented in Results section.

6) Bover-Cid, S; Serra-Castelló, C; Dalgaard, P; Garriga, M; Jofré, A. (2019). *New insights on Listeria monocytogenes growth in pressurized cooked ham: A piezo stimulation effect enhanced by organic acids during storage*. International Journal of Food Microbiology, 290, pp. 150-258. DOI: https://doi.org/10.1016/j.ijfoodmicro.2018.10.008

   ISSN: 0168-1605, Impact Factor: 4.187, ranked 23/139 in the category of *Food Science & Technology* (1st quartile).

7) Serra-Castelló, C; Jofré, A; Bover-Cid, S. *Enhancing high pressure bacterial inactivation by modified atmosphere packaging: effect of exposure time and cooked ham formulation*. (In preparation, tentative title)

8) Serra-Castelló, C; Jofré, A; Torrents-Masoliver, B; Bover-Cid, S. *Impact of packaging systems and high-pressure processing on the shelf-life of commercial cooked ham*. (In preparation, tentative title)

9) Serra-Castelló, C; C.C.P. Costa, J; Jofré, A; Bolívar, A; Pérez-Rodríguez, F; Bover-Cid, S. (2022). *A mathematical model to predict the antilisteria bioprotective effect of Lactilactobacillus sakei CTC494 in vacuum packaged cooked ham.* International Journal of Food Microbiology, 363, 109491. DOI: https://doi.org/10.1016/j.ijfoodmicro.2021.109491

   ISSN: 0168-1605, Impact Factor: 5.911, ranked 31/144 in the category of *Food Science & Technology* (1st quartile)[2].

10) Serra-Castelló, C.; Desriac, N; Jofré, A; Belletti, N; Coroller, L; Bover-Cid, S. (2022). *Key factors determining the behaviour of pathogens in dry-cured ham after high-pressure processing.* Applied Sciences, 12(24), 12732. DOI: https://doi.org/10.3390/app122412732

    ISSN: 2076-3417, Impact Factor: 2.838, ranked 39/92 in the category of *Engineering, Multidisciplinary* (2nd quartile)[1].

11) Serra-Castelló, C; Jofré, A; Garriga, M; Bover-Cid, S. (2020). *Modeling and designing a Listeria monocytogenes control strategy for dry-cured ham taking advantage of water activity and storage temperature*. Meat Science, 165, 108131. DOI: https://doi.org/10.1016/j.meatsci.2020.108131

    ISSN: 0309-1740, Impact Factor: 5.209, ranked 26/143 in the category of *Food Science & Technology* (1st quartile).

12) Serra-Castelló, C; Bover-Cid, S; Garriga, M; Beck Hansen, T; Gunvig, A; Jofré, A. (2020). *Risk management tool to define a corrective storage to enhance Salmonella inactivation in dry fermented sausages.* International Journal of Food Microbiology, 346, 109160. DOI: https://doi.org/10.1016/j.ijfoodmicro.2021.109160

    ISSN: 0168-1605, Impact Factor: 5.277, ranked 25/143 in the category of *Food Science & Technology* (1st quartile).

[2] According to 2021 Journal Citation Reports

List of abbreviations

ALOP	Appropriate Level of Protection
a_w	Water activity
BIOHAZ	Biological Hazards (EFSA Panel of biological hazards)
BRC	British Retail Consortium
CAC	Codex Alimentarius Commission
CFU	Colony Forming Units
EU	European Union
ECDC	European Center for Disease Prevention and Control
EFSA	European Food Safety Authority
FAO	Food and Agricultural Organization
FBO	Food Business Operators
FDA	Food and Drug Administration
FSAI	Food Safety Authority of Ireland
FSANZ	Food Standards Australia New Zealand
FSIS	Food Safety and Inspection Service
FSO	Food Safety Objective
GL	Guidelines
GRAS	Generally Recognized As Safe
HPA	Health Protection Agency
HPP	High-Pressure Processing
ICMSF	International Commission on Microbiological Specifications of Foods
ILSI	International Life Sciences Institute
ISO	International Organization for Standardization
LAB	Lactic Acid Bacteria
MAP	Modified Atmosphere Packaging
MC	Microbiological Criteria
NACMCF	National Advisory Commitee on Microbiological Criteria for Foods
NZFSA	New Zealand Food Safety Authority
PC	Performance Criteria
PO	Performance Objective
RASFF	Rapid Alert System for Food and Feed
RTE	Ready-To-Eat
USA	United States of America
WHO	World Health Organization

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Abstract

The food safety challenges derived from the market demands towards convenient food, such as ready-to-eat meat products with extended durability, motivate food business operators to explore and adopt new intervention strategies to increase the microbiological safety of their products. This thesis was focused on assessing and **modelling the behaviour of *Listeria monocytogenes* and *Salmonella*** towards different intervention strategies with the aim to **develop decision support systems** to increase the microbiological safety and to extend the safe shelf-life of different RTE meat products. Several challenge tests and predictive microbiological approaches were applied to characterize the behaviour of *Listeria monocytogene*s and *Salmonella* in RTE meat products in response to **antimicrobial and post-lethality intervention strategies** aiming (i) to inhibit the growth of *L. monocytogenes* on cooked ham*,* including biopreservation and modified atmosphere packaging (MAP) and (ii) to inactivate *L. monocytogenes* and/or *Salmonella* in raw pet food, cooked ham, dry-cured ham and/or dry-fermented sausages, including high pressure processing (HPP) or corrective storage. Moreover, the potential interactions between HPP and antimicrobial strategies were also assessed.

Antimicrobial strategies for cooked ham affected different kinetic parameters of the *L. monocytogenes* growth, the magnitude of which was dependent on the strategy, the application dose and the product characteristics. The lag time increase and particularly the growth rate reduction achieved with **organic acids** (lactate and diacetate), resulted in a higher extension of the safe shelf-life (3.5 to 9 fold) compared to **MAP** (1.1 to 1.7-fold). The **bioprotective** culture *Latilactobacillus sakei* CTC494 reduced the maximum population density of *L. monocytogenes* and applied at a dose 5 log higher than the pathogen, totally inhibited its growth either at 2 °C and at a dynamic temperature profile ranging from 2 to 8 °C.

Regarding **HPP**, the inactivation of *L. monocytogenes* and *Salmonella* was strain dependent and selected strains for each pathogen were obtained to be used in HPP validation studies through challenge testing. Particularly, for cooked ham a versatile pool of *L. monocytogenes* strains (CTC1034, CTC1011 and Scott A) with different HPP inactivation curves were selected and, for raw pet food, a pool of *Salmonella* (CTC1022, GN0082 and GN0085) was determined to be the most resistant to HPP. Interestingly, *L. monocytogenes* surviving HPP was able to grow in cooked ham during the subsequent refrigerated storage, with a rate piezo-stimulated (i.e. increased) by HPP. Interactions between HPP and other intervention strategies were identified. This included an antagonistic interaction for *L. monocytogenes* in cooked ham when HPP was combined with lactate, resulting in a dose-dependent piezo-protective effect. The transcriptomic analysis pointed out that, in the presence of lactate, *L. monocytogenes* could upregulate mechanisms involved in 1,2 propanediol, ethanolamine and methionine synthesis, providing a fitness advantage to withstand HPP. Moreover, lactate also enhanced the piezo-stimulation effect exerted by HPP on the growth rate of *L. monocytogenes*. Both, piezo-protection and piezo-stimulation effects highlighted the disadvantage of combining HPP with lactate, as the safety improvement and extension of the safe-shelf life was smaller than when applied as single strategies. In contrast, with diacetate *L. monocytogenes* inactivation by HPP was increased and the subsequent growth was reduced. In the same line, acidulation of raw pet food with lactic acid enhanced the *Salmonella* inactivation by HPP, avoiding the recovery of sublethally injured cells after HPP and increasing the inactivation rate. The combination of HPP and MAP did not increase *L. monocytogenes* inactivation by HPP but reduced its subsequent growth.

The efficacy of the **corrective storage** in dry-cured meat products was enhanced by increasing the temperature. While for *L. monocytogenes* in dry-cured ham the lower the water activity (a_w) the higher the inactivation, for *Salmonella* in dry-fermented sausages, lower a_w protected the pathogen, while acid products enhanced the inactivation. A corrective storage of 7 days at room temperature could reduce by 1 log *L. monocytogenes* and *Salmonella* in dry-cured ham and dry-fermented sausages, respectively.

Decision support tools based on the **developed and validated predictive models** were provided to support stakeholders with the assessment and application of the studied intervention strategies to control *L. monocytogenes* and *Salmonella* and improve the safety of RTE meat products. For instance, they can be used to set the conditions and/or technological parameters to inhibit the growth of *L. monocytogenes* in cooked ham, achieve the desired reduction of *Salmonella* in raw pet food with HPP considering the addition of lactic acid and/or the implementation of a freezing storage as well as to design a corrective storage for *L. monocytogenes* in dry-cured ham and for *Salmonella* in dry-fermented sausages taking advantage of the product characteristics.

Resum

Els reptes en seguretat alimentària derivats de les demandes del mercat relatives a aliments en format de conveniència, com els productes carnis llestos per al consum de llarga vida útil, motiven els operadors d'empreses alimentàries a explorar i adoptar noves estratègies d'intervenció per augmentar la seguretat microbiològica dels seus productes. Aquesta tesi es va enfocar **a avaluació i modelització del comportament de *Listeria monocytogenes* i *Salmonella*** en resposta a diferents estratègies d'intervenció, amb l'objectiu de desenvolupar **eines de suport per a la presa de decisions** per augmentar la seguretat microbiològica i extendre la vida útil segura de diferents productes carnis llestos per al consum. Es van aplicar diferents enfocs basats en assajos d'inoculació i microbiologia predictiva per caracteritzar el comportament de *L. monocytogenes* i *Salmonella* en resposta a **estratègies d'intervenció basades en l'aplicació antimicrobians i tractaments de post-letalitat** amb l'objectiu de (i) inhibir el creixement de *L. monocytogenes* en pernil cuit, inclosa la bioconservació i envasat en atmosfera modificada (MAP) i (ii) inactivar *L. monocytogenes* i/o *Salmonella* en aliments crus per a mascotes, pernil cuit, pernil curat en sec i/o salsitxes fermentades en sec, inclòs el processament per alta pressió (HPP) o l'emmagatzematge correctiu. A més, també es van avaluar les possibles interaccions entre HPP i les estratègies antimicrobianes.

Les estratègies antimicrobianes aplicades en pernil cuit van afectar diferents paràmetres cinètics del creixement de *L. monocytogenes*, la magnitud dels quals va ser dependent de l'estratègia, la dosi d'aplicació i les característiques del producte. L'augment de la fase de latència i, en particular, la reducció de la taxa de creixement assolida amb **àcids orgànics** (lactat i diacetat), va donar com a resultat una major extensió de la vida útil segura (3.5 a 9 vegades) en comparació amb **MAP** (1.1 a 1.7 vegades). El cultiu **bioprotector** *Latilactobacillus sakei* CTC494 va reduir la màxima densitat poblacional de *L. monocytogenes* i aplicat a una dosi 5 log superior a la del patogen, va inhibir totalment el seu creixement a 2 °C i a un perfil dinàmic de temperatura entre 2 i 8 °C.

En el cas del processament per **HPP**, la inactivació de *L. monocytogenes* i *Salmonella* depenia de la soca i es van obtenir soques seleccionades per a cada patogen per ser utilitzades en estudis de validació de HPP mitjançant assajos d'inoculació. Concretament, per al pernil cuit es va seleccionar un còctel versàtil de soques de *L. monocytogenes* (CTC1034, CTC1011 i Scott A) amb diferents corbes d'inactivació d'HPP i, per a aliments crus per a mascotes, es va determinar que el còctel de soques de *Salmonella* (CTC1022, GN0082 i GN0085) era el més resistents a HPP. Curiosament, la *L. monocytogenes* que va sobreviure a HPP va poder créixer en pernil cuit durant el posterior emmagatzematge refrigerat, amb una taxa de creixement piezoestimulada (és a dir, augmentada) per HPP. S'han identificat interaccions entre HPP i altres estratègies d'intervenció. Això va incloure una interacció antagònica per a *L. monocytogenes* en pernil cuit quan HPP es va combinar amb lactat, la qual cosa va resultar en un efecte piezoprotector dependent de la dosi. L'anàlisi transcriptòmica va assenyalar que, en presència de lactat, *L. monocytogenes* podria regular a l'alça els mecanismes implicats en la síntesi de 1,2 propanodiol, etanolamina i metionina, cosa que podria proporcionar un avantatge metabòlic per resistir l'HPP. A més, el lactat també va millorar l'efecte piezoestimulador exercit per AP sobre la taxa de creixement de *L. monocytogenes*. Tant els efectes de piezoprotecció com de piezoestimulació van destacar el desavantatge de combinar HPP amb lactat, ja que la millora de la seguretat i l'extensió de la vida útil segura van ser menors que quan es van aplicar com a estratègies úniques. Per contra, amb diacetat es va augmentar la inactivació de *L. monocytogenes* per HPP i es va reduir el creixement posterior. A la mateixa línia, l'acidulació d'aliments crus per a mascotes amb àcid làctic va millorar la inactivació de *Salmonella* per HPP, evitant la recuperació de cèl·lules subletalment danyades després d'HPP i augmentant la taxa d'inactivació. La combinació d'HPP i MAP no va augmentar la inactivació de *L. monocytogenes* per HPP, però en va reduir el creixement posterior.

L'eficàcia de l'**emmagatzematge correctiu** en productes carnis crus curats es va millorar augmentant la temperatura. Mentre que per a *L. monocytogenes* en pernil curat a menor activitat d'aigua (a_w) major inactivació, per a *Salmonella* en embotits fermentats, menor a_w protegia el patogen, mentre que els productes àcids potenciaven la inactivació. Un emmagatzematge correctiu de 7 dies a temperatura ambient podria reduir en 1 log *L. monocytogenes* i *Salmonella* en pernil curat i embotits fermentats, respectivament.

Es van **proporcionar eines de suport a la presa de decisions basades en els models predictius desenvolupats i validats** per donar suport a les parts interessades en l'avaluació i l'aplicació de les estratègies d'intervenció estudiades per controlar *L. monocytogenes* i *Salmonella* i millorar la seguretat dels productes carnis llestos

per al consum. Per exemple, es poden utilitzar per establir les condicions i/o paràmetres tecnològics per inhibir el creixement de *L. monocytogenes* en pernil cuit, aconseguir la reducció desitjada de *Salmonella* en aliments crus per a mascotes amb HPP considerant l'addició d'àcid làctic i/o la implementació d'un emmagatzematge en congelació així com dissenyar un emmagatzematge correctiu per a *L. monocytogenes* en pernil curat i per a *Salmonella* en embotits secs fermentats aprofitant les característiques del producte.

Resumen

Los desafíos en seguridad alimentaria derivados de las demandas del mercado relativos a alimentos convenientes, como los productos cárnicos listos para el consumo y de mayor durabilidad, motivan a los operadores de empresas alimentarias a explorar y adoptar nuevas estrategias de intervención para aumentar la seguridad microbiológica de sus productos. Esta tesis se focalizó en evaluar y **modelizar el comportamiento de *Listeria monocytogenes* y *Salmonella*** en respuesta a diferentes estrategias de intervención con el objetivo de desarrollar **herramientas de apoyo para la toma de decisiones** para aumentar la seguridad microbiológica y extender la vida útil segura de diferentes productos cárnicos listos para el consumo. Se realizaron ensayos de inoculación y se aplicaron distintos enfoques de microbiología predictiva para caracterizar el comportamiento de *Listeria monocytogenes* y *Salmonella* en respuesta a **estrategias de intervención antimicrobiana y postletales** aplicadas con el objetivo de (i) inhibir el crecimiento de *L. monocytogenes* en jamón cocido, incluida la bioconservación y envasado en atmósfera modificada (MAP) y (ii) para inactivar *L. monocytogenes* y/o *Salmonella* en alimentos crudos para mascotas, jamón cocido, jamón curado y/o embutidos fermentados, incluido el procesado por alta presión (HPP) o el almacenamiento correctivo. Además, también se evaluaron las posibles interacciones entre HPP y las estrategias antimicrobianas.

Las estrategias antimicrobianas aplicadas en jamón cocido afectaron diferentes parámetros cinéticos del crecimiento de *L. monocytogenes*, cuya magnitud fue dependiente de la estrategia, la dosis de aplicación y las características del producto. El aumento de la fase de latencia y, en particular, la reducción de la tasa de crecimiento lograda con **ácidos orgánicos** (lactato y diacetato), dio como resultado una mayor extensión de la vida útil segura (3.5 a 9 veces) en comparación con **MAP** (1.1 a 1.7 veces). El cultivo **bioprotector** *Latilactobacillus sakei* CTC494 redujo la máxima densidad poblacional de *L. monocytogenes* y aplicado a una dosis 5 log superior a la del patógeno, inhibió totalmente su crecimiento a 2 °C y a un perfil dinámico de temperatura entre 2 y 8 °C.

En cuanto a **HPP**, la inactivación de *L. monocytogenes* y *Salmonella* dependió de la cepa por lo que se seleccionaron and caracterizaron cepas de cada patógeno para ser utilizadas en estudios de validación de HPP mediante ensayos de inoculación. En particular, para el jamón cocido se seleccionó un cóctel versátil de cepas de *L. monocytogenes* (CTC1034, CTC1011 y Scott A) con diferentes curvas de inactivación por HPP y, para alimentos crudos para mascotas, se determinó que el cóctel de cepas de *Salmonella* (CTC1022, GN0082 y GN0085) era el más resistente a HPP. Curiosamente, la *L. monocytogenes* que sobrevivió a HPP pudo crecer en jamón cocido durante el posterior almacenamiento refrigerado, con una tasa piezoestimulada (es decir, aumentada) por HPP. Se identificaron interacciones entre HPP y otras estrategias de intervención. Esto incluyó una interacción antagónica para *L. monocytogenes* en jamón cocido cuando HPP se combinó con lactato, lo que resultó en un efecto piezoprotector dependiente de la dosis. El análisis transcriptómico señaló que, en presencia de lactato, *L. monocytogenes* podría regular al alza los mecanismos implicados en la síntesis de 1,2 propanodiol, etanolamina y metionina, lo que proporciona una ventaja metabólica para resistir la HPP. Además, el lactato también mejoró el efecto piezoestimulador ejercido por AP sobre la tasa de crecimiento de *L. monocytogenes*. Tanto los efectos de piezoprotección como de piezoestimulación destacaron la desventaja de combinar HPP con lactato, ya que la mejora de la seguridad y la extensión de la vida útil segura fueron menores que cuando se aplicaron como estrategias únicas. Por el contrario, con diacetato se aumentó la inactivación de *L. monocytogenes* por HPP y se redujo el crecimiento posterior. En la misma línea, la acidulación de alimentos crudos para mascotas con ácido láctico mejoró la inactivación de *Salmonella* por HPP, evitando la recuperación de células subletalmente dañadas después de HPP y aumentando la tasa de inactivación. La combinación de HPP y MAP no aumentó la inactivación de *L. monocytogenes* por HPP pero redujo su crecimiento posterior.

La eficacia del **almacenamiento correctivo** en productos cárnicos curados y embutidos fermentados se incrementó aumentando la temperatura. Mientras que para *L. monocytogenes* en jamón curado a menor actividad de agua (a_w) mayor inactivación, para *Salmonella* en embutidos fermentados, menor a_w protegía al patógeno, mientras que los productos ácidos potenciaban la inactivación. Un almacenamiento correctivo de 7 días a temperatura ambiente podría reducir en 1 log *L. monocytogenes* y *Salmonella* en jamón curado y embutidos fermentados, respectivamente.

Se **proporcionaron herramientas de apoyo a la toma de decisiones basadas en los modelos predictivos desarrollados y validados** para apoyar a las partes interesadas en la evaluación y aplicación de las estrategias de intervención estudiadas para controlar *L. monocytogenes* y *Salmonella* y mejorar la

seguridad de los productos cárnicos listos para el consumo. Por ejemplo, se pueden utilizar para establecer las condiciones y/o parámetros tecnológicos para inhibir el crecimiento de *L. monocytogenes* en jamón cocido, lograr la reducción deseada de *Salmonella* en alimentos crudos para mascotas con HPP considerando la adición de ácido láctico y/o la implementación de un almacenamiento en congelación así como diseñar un almacenamiento correctivo para *L. monocytogenes* en jamón curado y para *Salmonella* en embutidos secos fermentados aprovechando las características del producto.

INTRODUCTION

1. Introduction

1.1. Ready-to-eat meat products

The worldwide production of meat has been continuously increasing over the last decade with exceeding production values of 337 million tonnes since 2018 (Eurocarne, 2022). In the European framework, meat production in 2021 resulted in 62 and 43 million revenue streams from exportations and importations, respectively, which highlighted meat production as an engine for economic growth in Europe. Spain was reported to be the seventh country in meat production in 2021 after China, the United States of America, Brazil, Russia, Germany, and Mexico. The production of meat in Spain has been continuously increasing over years, reaching production values of 7.7 million tonnes, and representing 28% of the total food production. Particularly, Spanish meat production includes the manufacturing of meat foods made from pork (67%), beef (9%), poultry (2%) and sheep (2%). According to classifications included in the European Commission (2011) and the European Parliament and the Council of the European Union (2004; 2008), foods made of meat, i.e., edible parts of animals (included blood), can be classified as:

1. Fresh meat, i.e., meat that has not undergone any process other than chilling/freezing or packed under vacuum or modified atmosphere packaging (MAP) conditions.
2. Meat preparations, i.e., fresh meat with foodstuffs, seasonings or additives added (unprocessed products) and processed meat that has been reduced to fragments, which has undergone processes insufficient to modify the internal muscle fibre structure of the meat and thus to eliminate characteristics of fresh meat.
3. Meat products, i.e., processed meat that has undergone processes sufficient to modify the characteristics of fresh meat, so that the cut of the surface of the piece shows that product no longer has the characteristics of fresh meat. This category includes non-heat-treated and heat-treated processed meat, casings, coatings or decorations for meat and traditional immersion and/or dry-cured and dry-fermented products.

From overall meat foods, the consumption of meat at home mainly involves the consumption of fresh meat (Cruz, 2022). Particularly, consumers have shown an increased acceptance of frozen meat since 2019 probably due to habits acquired during COVID19 pandemic for the difficulties faced when shopping. Despite this, the relationship between meat consumption, lifestyle and a healthy and sustainable diet is a hot topic in social debate, and it is changing social trends. A change in consumer habits is being produced in the last years towards an increased demand of convenient and nutritionally improved ready-to-eat (RTE) meat foods, e.g., foods that require no or little preparation before their consumption, with an extended shelf-life, while at the same time being minimally processed (e.g., fresh appearance, without additives, etc.). This trend in consumer habits is expected to continue in the next years, with the global RTE meat products market growing at a compound annual rate of 4.8% until 2026, and with Europe and North America being the fastest growing and largest markets, respectively (Straits Research, 2023).
From the food safety perspective, RTE meat products are considered a food safety concern as they are intended to be consumed without the need for cooking or other processing effective to eliminate or reduce to an acceptable level the potential microorganism/s of concern (European Commission, 2005). In addition, the growing consumer demands and market trends of meat and meat products is creating new food technology and food safety challenges. Some food business operators have reformulated their RTE products to add “clean label” and “nutritional claims” that are recognized by the consumer and in line with initiatives of public health authorities to force food business operators to manufacture healthier foods (WHO, 2004). Clean labels are identified as an important market trend since consumers associate the term “clean label” with healthier products, i.e., products less processed and with the lowest number of additives

as possible (Asioli et al., 2017). However, in the case of the meat industry, some strategies implemented to add "clean labels" in foods are focused on alternatives to reduce salt (sodium) or nitrites, which are recognized to have an antimicrobial effect (Fraqueza et al., 2021). Therefore, food business operators can face important challenges when manufacturing and commercializing RTE meat products that can be in turn enhanced when aiming to add "clean labels".

These new consumer needs are motivating food business operators and scientists to look for and adopt new strategies to increase the food safety and shelf-life of products, which can be a key factor to drive the market of RTE meat products during the next years. Particularly, meats and meat products have a high impact on microbial food safety (EFSA & ECDC, 2022) being one of the main food categories incriminated in alerts and/or withdrawals related to *Listeria monocytogenes* and *Salmonella* as described in the following sections.

1.2. *Listeria monocytogenes* and listeriosis

Listeria monocytogenes is a rod-shaped bacillus with a cell diameter of 0.4-0.5 µm and 1-2 µm length. It is a Gram-positive, facultative anaerobic, catalase positive and oxidase negative, non-spore-forming bacterium that uses peritrichous flagella for movement (Wagner & McLauchlin, 2008).

The resistance of *L. monocytogenes* to harsh environments is variable, which can be partially explained by different features shown by the pathogen. In this sense, at least 13 different serotypes of *L. monocytogenes* (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7) have been identified (Painset et al., 2019). These different serotypes have been further grouped into 4 lineages; I, II; III and IV with different genetic features, including traits conferring resistance to heavy metals (in some serotypes of lineage II) or traits conferring virulence (in some serotypes of lineage I) among others (Painset et al., 2019). The optimum growth temperature of *L. monocytogenes* is between 30 and 37 °C. However, due to its psychotropic nature it can grow in a wide range of temperatures i.e., from -2 to 45 °C (Hudson et al., 1994). Besides temperature, *L. monocytogenes* is also able to grow in a wide range of pH (4.39 to 9.4) and water activity (a_w, 0.92 to 0.99) values (ICMSF, 1996) and in culture media formulated with up to 10% of salt (Wagner & McLauchlin, 2008). Some *L. monocytogenes* strains can withstand (although without growing) high concentrations of salt from 20 to 30%.

The distribution of *L. monocytogenes* in nature is ubiquitous. Through animals and vegetables, *L. monocytogenes* can enter processing plants and contaminate the food. The capability of *L. monocytogenes* to persist and survive in processing plants for long periods of time is mainly due to its ability to form and/or contribute to biofilms as well as its resistance to biocides used in the disinfection and cleaning protocols (Gómez et al., 2012) that confer protection in hostile environments such as those with a high concentration of salt, low concentration of oxygen or acidity.

Listeriosis is the zoonotic foodborne disease caused by *L. monocytogenes* affecting humans in two different forms as a function of *L. monocytogenes* infection: the non-invasive and the invasive form (WHO, 2018a). In the non-invasive form, the infective dose of the pathogen is estimated to be 10^7-10^9 cfu (McLauchlin et al., 2004). The main symptoms of the non-invasive form of *L. monocytogenes* infection are headache, fever, and gastrointestinal disorders such as vomiting, diarrhea or abdominal cramps, among others. On the other hand, the infective dose of *L. monocytogenes* in the invasive form is estimated to be 10^5-10^7 cfu, with risk groups (immunocompromised, pregnant women, infants, young children, and elderly people) having a 10- to 10,000-fold increased susceptibility compared to non-risk groups (Farber et al., 2021). The invasive form of *L. monocytogenes* infection can result in a high hospitalization and mortality rate. The main symptoms associated with this type of invasive form are septicaemia, meningitis, or encephalitis, among others. Moreover, it can be especially problematic in pregnant women, since if the infection is extended to foetus

it can result in important neurological disorders and problems during pregnancy that could lead to stillbirth (FAO & WHO, 2022).

Over the last 15 years, the number of invasive listeriosis reported cases in Spain have been increased, reporting the maximum number of cases in 2019 (Figure 1). In 2020-2021, a considerably decrease in the number of cases of listeriosis compared to previous years was reported, though it could be partially explained because in 2020-2021 the data reported by the different regions of Spain was not complete due to COVID-19 situation (EFSA & ECDC, 2022). Despite this, invasive listeriosis causes the highest proportion of hospitalized cases and deaths of all zoonoses in Europe, representing more than 50% of the deaths associated with foodborne diseases (EFSA & ECDC, 2022). The incubation time can be very variable from few hours up to 70 days (FAO & WHO, 2022), which hinders the implication of *L. monocytogenes*.

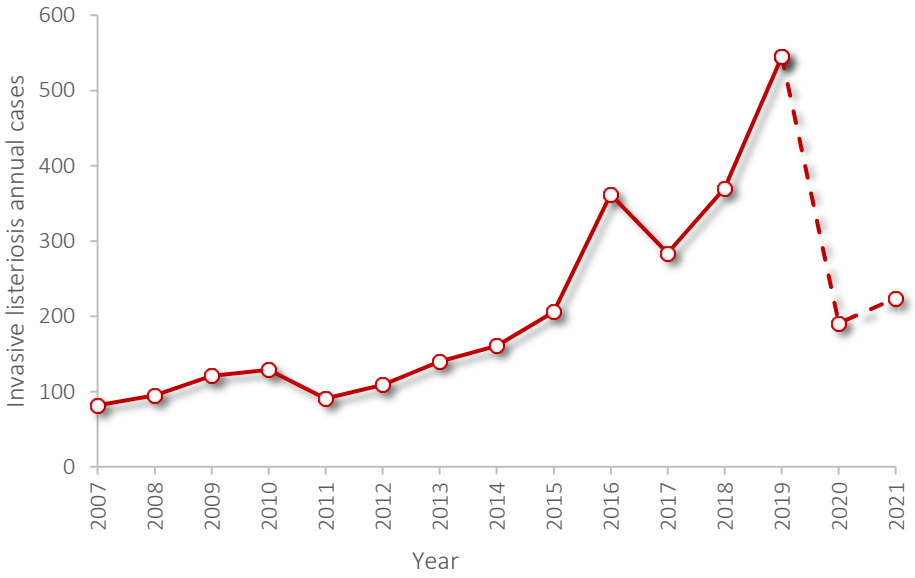

Figure 1. Number of reported invasive listeriosis annual cases in Spain by EFSA and ECDC (2022) annual reports (modified from Bover-Cid et al. (2022)). Dashed lines indicate incomplete data reported by Spain due to COVID-19 situation.

Listeriosis cases can be related with the consumption of a wide variety of RTE foods.

In this framework, results from quantitative microbial risk assessments performed by EFSA BIOHAZ Panel (2018) & FDA (2003) showed that deli meats (cooked meat products) were the RTE food category with a higher risk of listeriosis per annum compared to other RTE food categories (Figure 2).

The diversity and distribution of the pathogen can change along the production and distribution chains. Martín et al. (2014) found that the major proportion of *L. monocytogenes* isolates from meat processing plants had serotype 1/2a and 1/2c, which was in line with the overrepresentation of *L. monocytogenes* serotypes with the antigenic group 1/2 in food isolates (Vázquez-Boland et al., 2001). Despite this, most human listeriosis outbreaks have been related to *L. monocytogenes* serotype 4b (De Cesare et al., 2001; Salcedo et al., 2003), which could indicate that even the lower proportion of this serotype in processing plants and foods, its potential specific virulence may confer advantage towards other serotypes to invade the host.

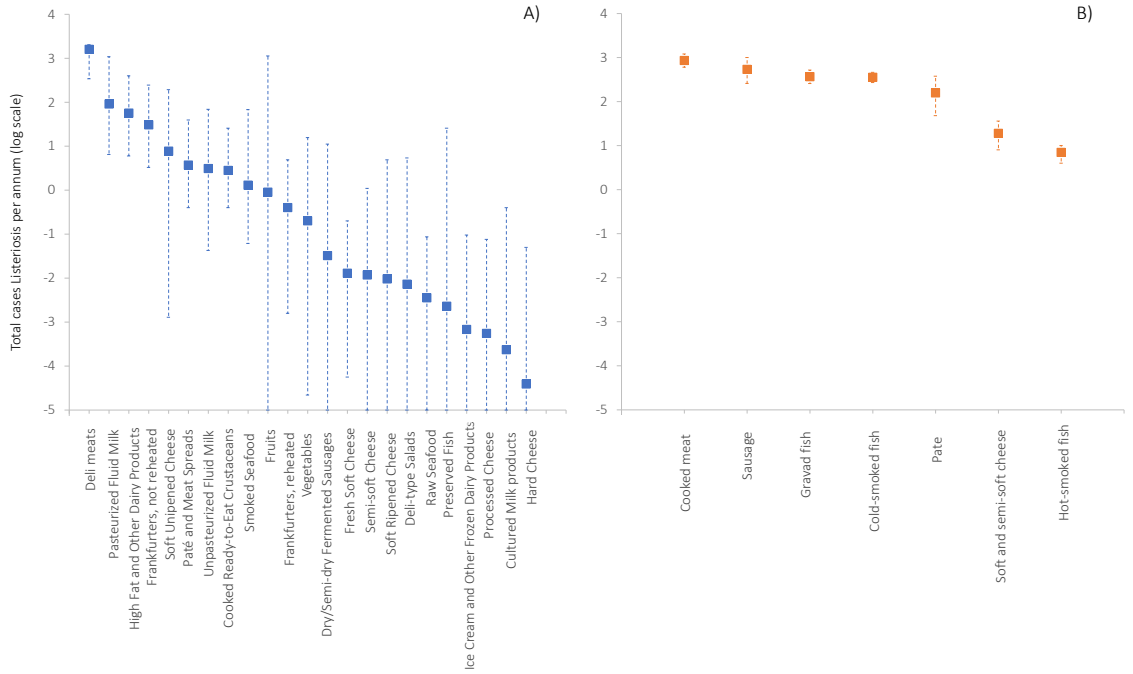

Figure 2. Predicted cases of Listeriosis (log scale) associated with RTE food categories for the total United States (A) and European (B) population on a per annum basis reported by EFSA BIOHAZ Panel (2018) & FDA (2003). In A, dots represent the mean and lines the confidence interval at 95%. In B, dots represent the median and lines the confidence interval at 97.5%.

The latest notifications in the Rapid Alert System for Food and Feed (RASFF) for *L. monocytogenes* in RTE meat products are gathered in Figure 3, which includes alerts (some related to food outbreaks), information notifications or border rejections. A higher number of alerts for *L. monocytogenes* in cooked meat products is reported compared to dry-cured and dry-fermented meat products.

The contamination of RTE foods with *L. monocytogenes* can occur at several different stages before consumption due to the ability of the pathogen to persist in industrial environments (Martin et al., 2011). In this sense, the higher number of alerts for *L. monocytogenes* in cooked compared to dry-cured and dry-fermented meat products (Figure 3) could be linked to the contamination of the product in post-processing operations and/or the ability of the pathogen to grow in cooked meat products up to detectable levels (Martin et al., 2011). On the other hand, although dry-cured and dry-fermented meat products could also be contaminated by *L. monocytogenes* during post-processing operations, the pathogen may tend to inactivate during the shelf-life due to the intrinsic characteristics of the product, resulting in a low number of alerts (Martin et al., 2011).

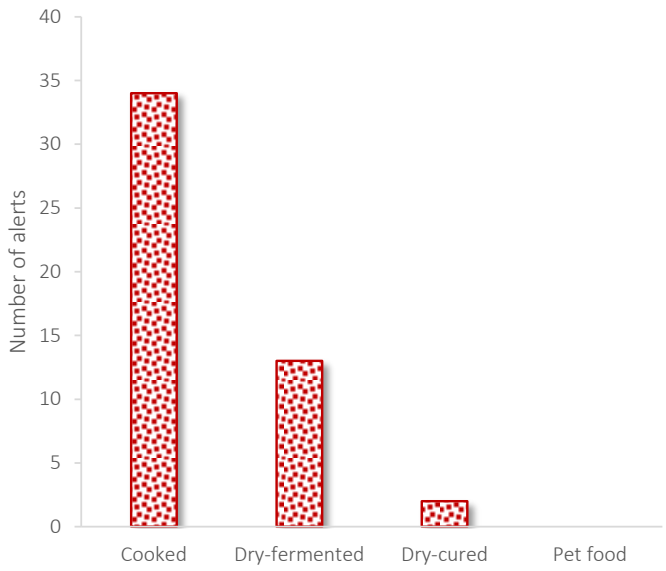

Figure 3. Notifications published in the European Rapid Alert System for Food and Feed (RASFF) regarding *L. monocytogenes* in different types of RTE meat products during 2020-2022.

1.3. *Salmonella enterica* and salmonellosis

Salmonella enterica is a rod-shaped bacillus of cell diameter of 0.7 to 1.5 µm and length of 2 to 5 µm. It is a Gram-negative, facultative anaerobic, non-spore-forming bacterium that uses peritrichous flagella for movement. The optimum growth temperature for *Salmonella* is 35-45 °C (ICMSF, 1996). However, it can grow in the range of temperatures between 5.2 and 46.2 °C (ICMSF, 1996). *Salmonella* is not able to grow at temperatures below 5.2 °C but it can persist and even grow in a wide range of pH (3.8-9.5) and a_w (0.93-0.99) values (ICMSF, 1996). Moreover, it can survive in low moisture foods (ICMSF, 1996).

S. enterica has more than 2600 different serotypes differentiated by their flagellar, carbohydrate and lipopolysaccharide structures, which can be divided into typhoidal and non-typhoidal *Salmonella*, the causative agents of typhoid/paratyphoid fevers and salmonellosis, respectively (Achtman et al., 2012).
The most common symptoms for salmonellosis are marked by acute onset, abdominal pain, diarrhoea, vomiting, diarrhoea, nausea and/or abdominal cramps (WHO, 2018b). Currently, the main non-typhoidal *Salmonella* serovars related with salmonellosis in humans are *S.* Enteritidis, *S.* Typhimurium and the monophasic variant of *S.* Typhimurium (EFSA & ECDC, 2022). In animals, the most common isolated non-typhoidal *Salmonella* serotypes are *S*. Enteritidis, *S*. Infantis, *S*. Typhimurium, the monophasic variant of *S*. Typhimurium and *S*. Derby (EFSA & ECDC, 2022). The infectious dose of non-typhoidal *Salmonella* is reported to be $5 \cdot 10^5$ cells and the symptoms for salmonellosis occur 6 to 72h after the consumption of food contaminated with *Salmonella* (Coburn et al., 2007). In the absence of treatment, symptoms usually last up to 7 days and resolve spontaneously. However, humans with cytokine abnormalities can be more susceptible to non-typhoidal *Salmonella* infections, and in these particular cases, non-typhoidal *Salmonella* serovars can cause severe extraintestinal diseases.
Over the last 5 years, the number of salmonellosis cases reported in Spain decreased (Figure 4). Data reported showed that in 2020-2021 this tendency has been stabilized, although this tendency could be not representative of reality since Spain did not receive data from all the regions that normally report due to COVID-19 situation (EFSA & ECDC, 2022). Moreover, salmonellosis was the second most reported zoonotic infection in humans after campylobacteriosis and *Salmonella* remained the commonest cause of foodborne illness outbreaks (about one in five) in Europe during 2021 (EFSA & ECDC, 2022).

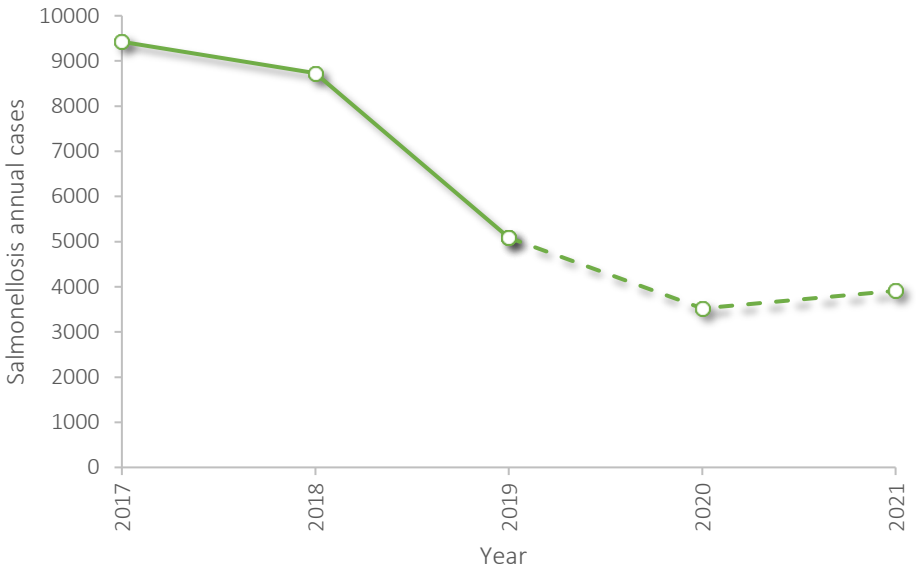

Figure 4. Number of reported salmonellosis annual cases in Spain by EFSA and ECDC (2022). Dashed lines indicate incomplete data reported by Spain due to COVID-19 situation.

Meat and meat products are usually reported as the second major type of products (after eggs/derived eggs products and dairy products) linked to foodborne outbreaks involving *Salmonella* (EFSA & ECDC, 2021).

In the framework of meat production, the presence of *Salmonella* in meat products is generally associated with contamination of raw materials as carcasses can be contaminated on the surface during slaughtering since animal skin and the digestive tract can host *Salmonella*. Food manipulated with poorly hygienic conditions is also a risk factor for *Salmonella* contamination. Raw RTE meat products such as dry-fermented sausages and raw pet food were associated with the highest alerts for *Salmonella* during 2020-2022 compared to RTE cooked meat products (Figure 5). Therefore, the presence of *Salmonella* in raw RTE meat products is particularly linked to, though not exclusively, the initial contamination of the raw meat materials, spices, and ingredients.

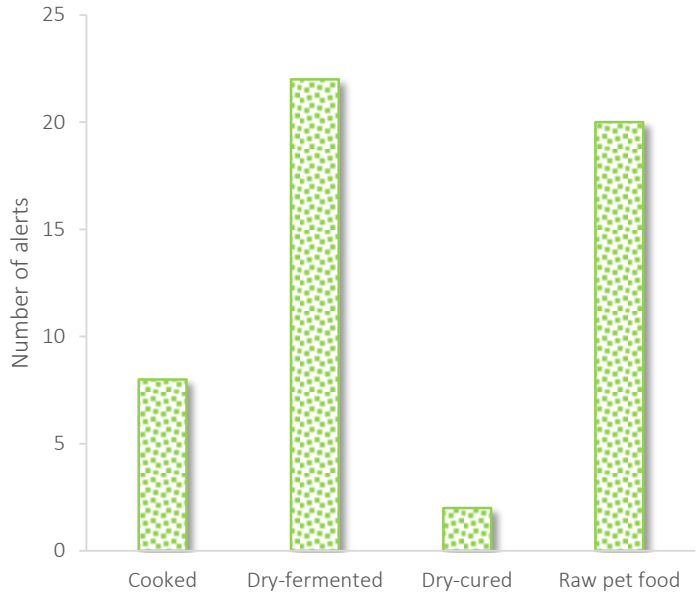

Figure 5. Notifications published in the RASFF portal regarding *Salmonella* in different RTE meat products during 2020-2022.

1.4. Microbiological food safety framework for RTE meat products

Risk analysis is the current regulatory framework applied in EU to elaborate and provide a systematic methodology based on science-based procedures for the determination of effective, proportionate and targeted measures to protect health considering available resources and attendant uncertainties (European Parliament & the Council of the European Union, 2002). Risk analysis consists of three interconnected components, namely risk assessment, risk management and risk communication (CAC, 1997, 2006).

1. **Risk assessment**: a scientifically based methodology aiming to provide risk managers with objective data on the health risk and its causes at specific time point. In microbiological food safety field, risk assessments include (FAO & WHO, 2021):
   - 1.1. (i) Hazard identification: a process for the identification of the microbial hazards (microorganisms and/or microbial toxins) of concern in food, and their associations with specific food commodities and certain high-risk groups in the population.
   - 1.2. (ii) Hazard characterization: a process describing the adverse effects that may result from the ingestion of the microbial hazard for different subpopulations, including risk groups. When possible, it must include a quantitative dose-response relationship.
   - 1.3. (iii) Exposure assessment: a qualitative and/or quantitative evaluation of the likely intake of microbial hazards due to the consumption of a specific food. The exposure assessment identifies the likelihood and level of the microbial hazard in a portion of food (i.e., concentration) as well as the amount of food (i.e., serving size to derive the dose of hazard ingested) for a given (sub)population in a specific period and may combine the information to estimate the exposure to the hazard. The exposure assessment should detail the various steps of the food chain pathway and the effect of relevant steps or processes can be assessed. This can be very powerful information for assessing risk management options.
   - 1.4. (iv) Risk characterization: the estimation of the probability and severity of the adverse effects that occur in a given (sub)population. This risk is estimated through the integration of results from the hazard identification, hazard characterization and

exposure assessment steps. These results provide science-based data to help risk managers to design and implement strategies to control food safety risks.

2. **Risk management**: is the process of weighing policy alternatives considering the assessed risk for the health protection of consumers and the promotion of fair-trade practices by selecting, implementing, and monitoring suitable options to accept, minimize or reduce the risk.

3. **Risk communication**: the interactive exchange of information and opinions throughout the risk analysis process concerning risk, risk-related factors, and risk perceptions, among risk assessors, risk managers, consumers, industry, the academic community, and others that are likely to be affected by risk management decisions.

To implement the knowledge provided by risk analysis at operational level, new food safety precepts were established to translate governmental policy for the protection of public health from foodborne diseases into targets for the control of hazards in the food chain (ICMSF, 2010; van Schothorst, 1998). The precepts included in the microbiological food safety system are:

1 Appropriate level of protection (ALOP): is the acceptable level of risk or tolerable risk. It can be a public health goal or an aim to reduce illness by a particular amount.
2 Food safety objective (FSO): the maximum level (prevalence or concentration) of a microbial hazard in the food at the time of consumption that contributes to the ALOP. FSO should be set by a competent authority based on a governmental risk assessment and an ALOP.
3 Performance objective (PO): the maximum level (prevalence or concentration) of a microbial hazard in a food at a specified step in the food chain (before the time of consumption) that contributes to an FSO and/or ALOP.
4 Performance criteria (PC): change in the hazard level achieved by the application of one or more control measures and required to meet the PO and/or FSO.
5 Microbiological criterion (MC): defines the acceptability of a product or food lot, based on the presence, absence, or level of microorganisms per unit mass, area, or lot.

Codex Alimentarius guidelines (CAC/GL-21, 1997) state the principles for the establishment and application of microbiological criteria for foods. In this framework, microbiological criteria must state (i) the microorganism(s) of concern and/or their toxins/metabolites, (ii) the detection and/or quantitative analytical methods to be used, (iii) a microbiological sampling plan defining the number of samples to be analysed and the size of the analytical unit, (iv) the microbiological limits at a specified point of the food chain and (v) the number of analytical samples that should conform these limits. Moreover, the microbiological criteria should state the food and the point in the food chain where the criteria applies and the actions to be undertaken when the microbiological criterion is not met (CAC/GL-21, 1997).

It is important to highlight that there is a separation of responsibilities when it comes to establishing these precepts. While ALOPs, FSOs and MC can only be set by governments, POs and PCs may be set by the food business operators as tools that help to meet the target FSO. The implementation of this microbial risk system allows to determine in a more effective way the activities and control measures that food business operators must conduct to guarantee the food safety of products that they produce and commercialize, considering the impact on health and protection for consumers (Gorris, 2005).

To minimize the risk for consumers, regulations for microbiological criteria in relation to pathogen contamination of RTE meat products are implemented in many countries and regions (EFSA, 2007). Microbiological criteria indicate the sampling plan and analytical methodology to be followed by food

business operators to determine the acceptability of foodstuffs according to each food category and pathogen. Moreover, some microbiological criteria also refer to the measures to be followed by food business operators when unacceptable foodstuffs are obtained (Gorris, 2005).

Nevertheless, microbiological criteria and their requirements can vary among the different countries, including differences in the categorization of food, sampling plans, analytical methods, the measures to be applied for unacceptable foodstuffs and/or in the time-framework of the food production where the microbiological criteria apply as described in the next sections.

1.4.1 Microbiological criteria for *L. monocytogenes*

The microbiological criteria applied to *L. monocytogenes* for RTE meat products in different countries are gathered in Table 1. Europe (European Commission, 2005), Canada (Health Canada, 2011) and Australia and New Zealand (FSANZ, 2014a) establish different limits according to the ability of *L. monocytogenes* to grow in the product in line with the Codex Alimentarius Commission (CAC/GL-21, 1997).
RTE foods not able to support the growth of *L. monocytogenes* are defined as those with pH ≤ 4.4, with a_w ≤ 0.92, with pH ≤ 5 and a_w ≤0.94, or with a refrigerated shelf-life of ≤ 5 days. Alternatively, RTE foods with other characteristics can be also defined as not able to support the growth of *L. monocytogenes* if it can be scientifically justified. In addition, the microbiological criteria applied in Canada and Australia and New Zealand also include in this group frozen foods (i.e., foods consumed frozen and those intended to be thawed immediately before consumption) and foods in which the level of *L. monocytogenes* will not increase more than 0.5 log for at least the stated shelf-life (FSANZ, 2014a; Health Canada, 2011). For RTE products not able to support the growth of *L. monocytogenes*, a maximum level of 100 cfu/g of the pathogen is allowed during the product's shelf-life (European Commission, 2005; FSANZ, 2014b; Health Canada, 2011).

In the case of RTE meat products (other than those intended for infants and special medical purposes) able to support the growth of the pathogen, a general maximum level of 100 cfu/g of *L. monocytogenes* is allowed during the product's shelf-life if food business operators can demonstrate to the satisfaction of the competent authority that the product will not exceed the limit of 100 cfu/g throughout the shelf-life (European Commission, 2005; FSANZ, 2014b; Health Canada, 2011). In case of Canada, these microbiological criteria are also applied in RTE foods that are known to occasionally contain low levels of *L. monocytogenes* and do not have a kill step and/or have a stated shelf-life at refrigeration temperatures of ≤ 5 days (Health Canada, 2011).

Alternatively, when food business operators cannot provide data to prove to the satisfaction of the competent authority that the product will not exceed the limit of 100 cfu/g throughout the shelf-life, the no detection of the pathogen in 25 g of product is required before the food has left the immediate control of the food business operator (European Commission, 2005; FSANZ, 2014b; Health Canada, 2011).

In the United States and China, the microbiological criteria applied for *L. monocytogenes* is set up under the zero-tolerance policy, being more restrictive and requiring the no detection of *L. monocytogenes* in 25 g of product regardless of the ability of the product to support or not the growth of the pathogen (FSIS, 2014; GB 29921, 2013).

Table 1. Microbiological criteria for *L. monocytogenes* applied in RTE meat products (other than poultry products) for human consumption.

Category of product	Sampling plan[a]		Limits[b]	Application
	n	c	m M	
Europe (European Commission, 2005)				
Ready-to-eat foods able to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes	5	0	100 cfu/g	Products placed on the market during their shelf-life[b]
			Not detected in 25 g	Before the food has left the immediate control of the food business operator, who has produced it[c]
Ready-to-eat foods unable to support the growth of *L. monocytogenes,* other than those intended for infants and for special medical purposes	5	0	100 cfu/g	Products placed on the market during their shelf-life
Ready-to-eat foods intended for infants and ready-to-eat for special medical purposes	10	0	Not detected in 25 g	Products placed on the market during their shelf-life
Canada (Health Canada, 2011)				
Ready-to-eat foods that can support the growth of *L. monocytogenes*	5	0	Not detected in 25 g	Products placed on the market throughout the stated shelf-life
Ready-to-eat foods in which limited growth of *L. monocytogenes* to levels not greater than 100 cfu/g can occur throughout the stated shelf-life	5	0	100 cfu/g	Products placed on the market throughout the stated shelf-life
Ready-to-eat foods in which the growth of *L. monocytogenes* cannot occur throughout the expected shelf-life of that food	5	0	100 cfu/g	Products placed on the market throughout the stated shelf-life
Australia and New Zealand (FSANZ, 2014a)				
Ready-to-eat food in which the growth of *L. monocytogenes* can occur	5	0	Not detected in 25 g	Products placed on the market throughout the stated shelf-life
Ready-to-eat food in which the growth of *L. monocytogenes* will not occur	5	0	100 cfu/g	Products placed on the market throughout the stated shelf-life
China (GB 29921, 2013)				
Ready-to-eat meat products (cooked and raw/cured)	5	0	Not detected in 25 g	NR
USA (FSIS, 2014)				
Ready-to-eat products where conditions reduce risk	5	0	Not detected in 25 g	NR
Ready-to-eat products where conditions reduce risk that are intended specifically for highly susceptible individuals	15	0	Not detected in 25 g	NR
Ready-to-eat products where conditions cause no change in concern	10	0	Not detected in 25 g	NR
Ready-to-eat products where conditions cause no change in concern that are intended specifically for highly susceptible individuals	30	0	Not detected in 25 g	NR
Ready-to-eat products where conditions increase concern	20	0	Not detected in 25 g	NR
Ready-to-eat products where conditions increase concern that are intended specifically for highly susceptible individuals	60	0	Not detected in 25 g	NR

NR: not reported

[a]n: number of units comprising the sample; c: number of sample units giving value over m or between m and M.

[b]m: microbiological level that separate conforming from non-conforming (defective) analytical units according to the sampling plan. M: microbiological criterion that separates conforming from non-conforming (defective) analytical units. In RTE meat products, m is equal to M for *L. monocytogenes*.

In this framework, the regulation 9CFR part 430 (*Listeria rule*) developed by the US Food Safety Inspection Service (FSIS) establishes an interesting approach to control *L. monocytogenes* in post-lethality exposed RTE meat products, e.g., RTE meat products susceptible to be contaminated with the pathogen. The *Listeria rule* requires that to control *L. monocytogenes*, establishments producing RTE meat products must comply with 1 of the 3 identified operating alternatives, which must be included in their HACCP plan and its effectiveness validated. These alternatives are designed to address the potential post-lethality contamination of *L. monocytogenes* in RTE products and consist of:

1. Alternative 1: to apply a post-lethality treatment to reduce or eliminate *L. monocytogenes* and to use an antimicrobial agent or process to suppress or limit the growth of the pathogen, in addition to sanitation standard operating procedures.
2. Alternative 2: use of either (a) a post-lethality treatment to reduce or eliminate *L. monocytogenes* or (b) an antimicrobial agent or process to suppress or limit the growth of the pathogen, in addition to sanitation standard operating procedures.
3. Alternative 3: to rely only in the sanitation standard operating procedures without the application of any additional specific control measure, e.g., post-lethality treatments or an antimicrobial agent/process.

The food business operators must validate (provide evidence) that the control measure applied reduces at least 1 log of *L. monocytogenes* to be considered a post-lethality treatment. In the same line, if the food business operator provides evidence that the control measure achieves the performance criteria of at least 2-log reduction or greater, it is considered that the application of this post-lethality treatment achieves an increased level of control of the pathogen. In the case of the use of antimicrobial agents or processes, food business operators must validate that *L. monocytogenes* could not grow more than 2 log units.
On the other hand, if establishments choose alternatives 2b or 3, the sanitation program must provide evidence that *L. monocytogenes* is not detected in the food contact surfaces of the processing environment, and they must be implemented according to the sampling plans described in Table 1. Moreover, when choosing alternative 3, establishments must verify the efficacy of the corrective actions taken with respect to sanitation after detecting *L. monocytogenes* on a food contact surface.

In the particular case of RTE foods that are detected to not fulfil microbiological criteria and that are not yet at retail level (e.g., a RTE meat product positive for *L. monocytogenes* or that has passed over a tested positive food contact surface), can be reprocessed by food business operators by applying treatments to eliminate the pathogen (European Commission, 2005; FSIS, 2012). In these cases, FSIS (2012) requires that the application of post-lethality treatments for reprocessing contaminated products should achieve at least a 5-log reduction of *L. monocytogenes*.

1.4.2 Microbiological criteria for *Salmonella*

The microbiological criteria applied to *Salmonella* in RTE meat products intended for human consumption are gathered in Table 2. In Europe, Australia and New Zealand, Canada, United States and China a "zero tolerance" policy for *Salmonella* is applied, requiring no detection of *Salmonella* in 25g of product along the product's shelf-life (European Commission, 2005; FSANZ, 2014b; FSIS, 2017; GB 29921, 2013; Health Canada, 2013). The main difference between the microbiological criteria applied in different countries is the stringency of the associated sampling plants. In the United States a higher number of RTE food samples (n=30-60) is required compared to other countries (n=5) (Table 2).

Some regulations and guidelines provide performance criteria to be achieved by control measures adopted/implemented by the food business operators to comply with the zero-tolerance policy for *Salmonella*.

Table 2. Microbiological criteria for *Salmonella* applied in RTE meat products (other than poultry products) for human consumption.

Category of product	Sampling plan[a]		Limits[b]	Application
	n	c	m M	
Europe (European Commission, 2005)				
Minced meat and meat preparations intended to be eaten raw	5	0	Not detected in 25 g	Products placed on the market during their shelf-life
Mechanically separated meat (MSM)	5	0	Not detected in 25 g	Products placed on the market during their shelf-life
Meat products intended to be eaten raw, excluding products where the manufacturing process or the composition of the product will eliminate the *Salmonella* risk	5	0	Not detected in 25 g	Products placed on the market during their shelf-life
Canada (Health Canada, 2013)				
Cooked RTE meat products	5	0	Not detected in 25 g	Products placed on the market during their shelf-life
Fermented and dried RTE meat products	5	0	Not detected in 25 g	Products placed on the market during their shelf-life
Australia and New Zealand (FSANZ, 2014b)				
Packaged cooked cured/salted meat	5	0	Not detected in 25 g	NR
All comminuted fermented meat that has not been cooked during the production process	5	0	Not detected in 25 g	NR
China (GB 29921, 2013)				
Ready-to-eat meat products (cooked and raw/cured)	5	0	Not detected in 25 g	NR
USA (FSIS, 2017)				
Foods that would not normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption	30	0	Not detected in 25 g	NR
Foods that would not normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption and are intended for consumption by the aged, the infirm and infants	60	0	Not detected in 25 g	NR

NR: not reported

[a]n: number of units comprising the sample; c: number of sample units giving value over m or between m and M.

[b]m: microbiological level that separates conforming from non-conforming (defective) analytical units according to the sampling plan. M: microbiological criterion that separates conforming from non-conforming (defective) analytical units. In RTE meat products, m is equal to M for *Salmonella*.

In this framework, guidelines from the United States (FSIS, 2017) and Canada (Health Canada, 2020) recommend that the time/temperature combination during the cooking step (thermal treatment) should provide enough heat to achieve at least 6.5 or 7-log reduction of *Salmonella* in cooked meat products not

containing and containing poultry, respectively. FSIS (2017) also recommends as a target 5-log reduction of *Salmonella* during the cooking process, but establishments should provide additional documental support for each lot demonstrating that levels of *Salmonella* are low enough to be controlled by a process achieving 5-log reduction with an appropriate safety margin. In this case, establishments should conduct a baseline study on the raw materials to demonstrate, with reasonable confidence, that less than 0.01% of the raw product contains > 10 cfu/g of *Salmonella* before cooking.
In case of RTE shelf-stable meat products (e.g., dried meat products), the recommendations to control *Salmonella* included in the guidelines from the United States (FSIS, 2017) and Canada (Health Canada, 2020) are focused on the control of *Escherichia coli* O157:H7 as a target microorganism in products containing beef, which are the most common type of RTE shelf-stable meat products found in the United States and Canada. According to the guidelines, it is recommended that establishments achieve a 5 and 7-log reduction of *Salmonella* in RTE shelf-stable meat products not containing and containing poultry, respectively (FSIS, 2017; Health Canada, 2020). Additionally, these guidelines provide five different options that can be used to control *Salmonella:* (i) to include as a part of the manufacturing a heat process recognized to control *Salmonella*; (ii) to use a manufacturing process (combination of fermentation, heating, holding and/or drying) which has already been scientifically validated to achieve 5 and 7-log reduction of *Salmonella* in products non containing and containing poultry, respectively; (iii) to hold and test each production lot, with the sampling plan being representative of the lot and with the analysis of 30 samples of 25 g of the finished product, (iv) to perform a microbiological testing program of raw meat and batter for *Salmonella* with the analysis of 15 samples of 25 g of the finished product as a part of the operator's HACCP process, which has been scientifically validated as achieving at least 2-log reduction of *Salmonella*; (v) to perform a validation challenge study to demonstrate that their manufacturing process achieve a 5 and 7-log reduction of *Salmonella* in products not containing and containing poultry, respectively (FSIS, 2017; Health Canada, 2020).

1.5. Intervention strategies to control pathogenic microorganisms

At operational level, the microbiological safety of foods can be managed at one or more steps along the production chain through the effective implementation of control measures, e.g., intervention strategies, which efficacy is validated to prevent, eliminate, or reduce a food safety hazards to a tolerable level. The control measures implemented by food business operators will influence the change (if any) between the incoming microbiological hazard level (H_0) at a particular level of the cold chain and the maximum microbiological hazard level of the food product at one step of the production and storage before consumption (Performance Objective, PO) or during consumption (Food Safety Objective, FSO). Control measures can be implemented with the objective of: (i) controlling the initial levels of microorganisms, (ii) eliminating or reducing to acceptable level the microorganisms and (iii) preventing or minimizing the re- or cross-contamination and growth of microorganisms in the food (ICMSF, 2002; ILSI, 2005). The required outcome of the control measure/s should achieve the performance criteria (PC) at one or more steps along the production chain. However, to ensure the safety of a particular food supply chain, food business operators must consider also other factors when determining the PC; including the initial levels of the target microorganism in their product at one or more steps along the cold chain and the variability associated with the processing.

In this framework, the conceptual equation proposed by the International Commission of Microbiological Specifications for Food (ICMSF, 2002) may be used to support decisions related to the design and evaluation of the efficacy of intervention strategies aiming to achieve a specific outcome such as the specified performance objective (PO) or food safety objective (FSO) (Eq. 1). Once validated, these intervention strategies can be implemented by food business operators as control measures.

$$H_0 - \sum R + \sum I \leq \text{PO or } FSO \qquad \text{Eq. 1}$$

Where the H_0 is the initial concentration of microorganism in the product; $\sum R$ is the reduction of microorganisms; $\sum I$ is the pathogen's increase (growth or cross-contamination) during the production and/or storage; *PO* is the performance objective and *FSO* is the food safety objective.

Food preservation implies submitting microorganisms in a hostile environment to reduce their growth, limit their survival and even inactivate them. The type and combination of factors associated with the food matrix (e.g., pH, a_w, antimicrobial compounds, endogenous microorganisms), the ones conferred by processing conditions (e.g., thermal treatments, post-lethality treatments) and/or storage (e.g., packaging, temperature) will determine how microorganisms will overcome these hostile environments and their level in the final product.
Different technologies can be applied to control microorganisms in food. Thermal technologies such as pasteurization or sterilization that have been proved to inactivate microorganisms in different food matrices and are used as the main lethal treatment in the processing step for the manufacturer of cooked meat products. However, heat treatments cannot be used for the hygienization of raw meat products such as raw pet food or dry-cured meat products due to the impact on the sensory characteristics. As an alternative, the use of non-thermal processing technologies as intervention strategies for RTE meat products has attracted attention for its potential in controlling pathogenic bacteria without significantly affecting quality traits of food (Farkas, 2016).
By understanding the impact of the different preservation factors and their interaction on microorganisms, the food safety management based on the hurdle technology concept can be developed. The hurdle technology concept relies on the intelligent combination of diverse preservative factors (hurdles/barriers) that microorganisms are not able to overcome (Leistner, 1978). Following this principle, food business operators can take advantage of the impact of their intrinsic and extrinsic factors of food and storage conditions on microorganisms and can reduce the intensity of the treatments (Leistner & Gorris, 1995). Thereby, the combination of non-thermal technologies with the intrinsic factors and storage conditions can enhance the food safety of RTE meat products while keeping the technological, sensorial, and nutritional properties of the food, which would match with the increasingly demanded minimally processed high quality RTE meat products with an extended shelf-life.

In the next sub-sections, non-thermal technologies that are often used as intervention strategies for RTE meat products are presented, including biopreservation and modified atmosphere packaging as strategies aimed to limit and/or inhibit the growth of microorganisms, and high pressure processing (HPP) as strategy aimed to reduce/inactivate the levels of microorganisms.

1.5.1 Biopreservation

Biopreservation is defined as the use of microorganisms and/or their antimicrobial metabolites to enhance microbiological safety and extend the shelf-life of food and with minimal impact on its sensory characteristics (Argyri et al., 2022). In case of meat products, biopreservation strategies based on the use of natural antimicrobials (e.g., organic acids and their salts, bacteriocins) synthesized by microorganisms such as lactic acid bacteria (lactic acid/lactate) or acetic acid bacteria (acetic acid/acetate/diacetate) (Hugas, 1998) are frequently applied nowadays.

1. Organic acids and their salts

The antimicrobial effect of organic acids is related to their undissociated form, which can freely permeate the cytoplasmatic membrane (Hirshfield et al., 2003). Once in the cytoplasm, they dissociate leading to an

accumulation of protons within the cell, which interfere with the metabolism of bacteria. Therefore, the antimicrobial effect of organic acids is enhanced in products with low pH because the concentration of the undissociated form increases as the pH decreases. Moreover, the different pKa of organic acids determine the amount of undissociated form depending on the pH, leading to a specific antimicrobial effect depending on the organic acid type. Specific effects for different types of organic acids have been reported, including the bacterial membrane disruption (leakage, transport mechanisms), the loss of active transport of nutrients through the membrane, the disruption of acid transport by alteration of cell membrane permeability, the inhibition of essential metabolic reactions, the accumulation of toxic anions, the energy stress response to restore homeostasis, the chelation as permeabilizing of outer membrane and metal ions, and the inhibition of other stress responses such as the heat-shock response.

2. Bacteriocins

Bacteriocins are a heterogenous group of bioactive proteins or peptides synthesized by bacteriocin-producing bacteria that have antimicrobial activity against other bacteria (Yang et al., 2014). The antimicrobial activity of bacteriocins is related to their combination with the corresponding receptor of the sensitive bacteria, which results in the death of the sensitive bacteria. Some antimicrobial activities of bacteriocins are pore-forming type, peptidoglycanase type or nuclease type with DNase and RNase function (Yang et al., 2014).

Bacteriocins could be used as an intervention strategy to prevent and/or limit the growth of pathogenic microorganisms if these natural antimicrobials have been previously approved by the competent authorities (Soltani et al., 2021).
On the one hand, bacteriocins can be used by food industry through the addition of bioprotective cultures (bacteriocin-producing bacteria) in the food. The psychotropic nature of some bacteriocin producing lactic acid bacteria with antilisteria activity makes these bioprotective cultures a versatile strategy to be used by industry to limit the growth and even compromise the viability of *L. monocytogenes* in raw and cooked meat products stored at refrigeration temperatures (Aymerich et al., 2006). However, these bioprotective cultures must have the Qualified Presumption of Safety (QPS) status according to the European Food Safety Authority (EFSA) or Generally Recognized as Safe (GRAS) to U.S. Food and Drug Administration to be used as food ingredients (Laulund et al., 2017).

On the other hand, bacteriocins can be also added in the meat products as (semi)purified bacteriocins or in the form of bioactive powders (De Vuyst & Leroy, 2007). However, the efficacy of bacteriocins against target microorganisms may be reduced compared to that observed with the addition of bioprotective cultures due their interaction with food matrices. Until now, only the nisin (E-234) has been approved has a bacteriocin to be used for food preservation in semolina and tapioca puddings (and similar products), ripened cheese and processed cheese, clotted cream, mascarpone and pasteurized liquid egg (European Commission, 2010). In this context, EFSA Panel on Food Additives and Nutrient Sources added to Food (2017) concluded in 2017 that the use of nisin in unripened cheese and heat-treated meat products at controlled levels would not be of safety concern. Besides this, there are some commercial bioactive powders, i.e., fermented products containing bacteriocin producers, such as Nisaplin® and MicroGard® fermentates (Dupoint) and DuraFresh products (Kerry), which are approved to be used as ingredients (Soltani et al., 2021).

1.5.2 Modified atmosphere packaging

Modified atmosphere packaging of RTE meats is mainly commonly used to extend their shelf-life by reducing (and even, inhibiting) the growth of spoilage microorganisms (Narasimha Rao & Sachindra, 2002). However, it can also be sued as a strategy to increase the safety of products by reducing the growth of pathogens. In this section, the two main modified atmosphere packaging techniques are described:

1. Vacuum packaging: is a preservation packaging technique that consists in removing the air from the interior of the packaging (leading to extreme low oxygen levels) before sealing the package by applying vacuum (Narasimha Rao & Sachindra, 2002). The depletion of almost all oxygen contributes to the inhibition of the growth of aerobic bacteria and to reduce the growth of facultative anaerobic bacteria. Besides controlling microbial growth, vacuum packaging can also offer an advantage in controlling oxidative rancidity.

2. Modified atmosphere packaging with CO_2: is a preservation packaging technique consisting in removing the air from the interior of the packaging and filling it with a combination of gases containing carbon dioxide (CO_2), nitrogen and/or oxygen before sealing the package (Narasimha Rao & Sachindra, 2002). CO_2 is recognized to have an antimicrobial effect, inhibiting the growth or even causing the death of some microorganisms (e.g., fungi, aerobic or facultative aerobic bacteria). The bacterial growth inhibition by CO_2 is reported to be proportional to the concentration of CO_2 dissolved in the water phase of the food matrix. The solubility of CO_2 is increased at low storage temperatures. Thereby, MAP containing CO_2 can be used as an intervention strategy to limit the growth of pathogenic microorganisms able to grow at refrigeration temperatures, such as *L. monocytogenes*, increasing the safe shelf-life of RTE meat products. Moreover, MAP containing CO_2 can extend the shelf-life by controlling oxidation.

1.5.3 High pressure processing

High pressure processing (HPP) or high hydrostatic pressure is a non-thermal technology cited as one of the best innovations in food processing from the last 60 years and the production of pressurized RTE meat products is increasing each year in response to the effort made by manufacturers to increase the safety of their products. Nowadays, the application of HPP as a post-packaging and/or post-lethality treatment in meat products counts for the 25-30% out of the total pressurised products in the market (Jung & Tonello-Samson, 2018).

The commercial use of HPP in the meat industry involves the pressurization of RTE meat products up to 600 MPa for a relatively short holding time (Bolumar et al., 2021). The impact of HPP on food follows three main principles. The first one is the Le Chatelier's principle stating that the application of pressure shifts an equilibrium towards the state that occupies a smaller volume (Balasubramaniam et al., 2015). The second one is the isostatic principle, stating that pressure is instantaneously and uniformly transmitted to food regardless of its size and geometry and the product retains its shape after decompression (Smelt, 1998). The third one is the microscopic ordering principle, stating that the increase of pressure enhances the molecular ordering of material at constant temperature (Agregán et al., 2021). All these principles have enabled the development of pressurized meat products with acceptable quality and safety traits to be successfully commercialized with a considerably reduced processing time compared to thermal treatments.

The impact of HPP on microorganisms depends on several factors including the pressure applied and the type of food matrix among others (Campus, 2010). Generally, the application of HPP at pressures > 200

MPa impairs cellular structures and processes, such as cell motility, cell division, nutrient uptake, ethanol fermentation, membrane protein function, replication, transcription, protein synthesis, protein oliogomerization, soluble enzyme function, viability, or protein structure, that can result in the death of microorganisms (Abe, 2007).

The efficacy of HPP in inactivating microorganisms in food has been proved in many studies (EFSA BIOHAZ Panel, 2022). However, it can be affected by several different factors including those related to microorganisms such as type of microorganism, taxonomic unit, strain and physiological state; factors related to HPP processing conditions such as pressure level, holding time and processing temperature as well as factors related with intrinsic (e.g., pH, a_w, redox potential, composition, etc.) and extrinsic (e.g. packaging, temperature, humidity, etc.) factors of the products.
Some of the studies that have evaluated the efficacy of HPP as an intervention strategy for *L. monocytogenes* and *Salmonella* in meat products are gathered in Table 3 and Table 4. Results from the studies show that pressure level, holding time and strain are key factors determining the inactivation of the pathogens by HPP. In addition, the recompilation of these studies raises up variable patterns between the different types of meat products on *L. monocytogenes* and *Salmonella* inactivation by HPP, indicating the important influence of the food matrix physicochemical characteristics. With the application of similar HPP treatments, microbial inactivation is higher in raw and cooked meat products compared to that observed in dry-cured and dry-fermented meat products after the curing, ripening and drying. This can be partially explained by the lower a_w of dry-cured and dry-fermented meat products compared to raw and cooked meat products (Bover-Cid et al., 2015, 2017).
Besides inactivation by HPP, these studies also show variability in the behaviour of *L. monocytogenes* and *Salmonella* after HPP, i.e., during the subsequent storage, depending on the type of meat product (Table 3).
In raw and cooked meat products, *L. monocytogenes* and *Salmonella* can survive and grow during the storage after HPP in most of the cases if they are stored at a favourable temperature (Table 3). This can be attributed to the physicochemical characteristics of raw and cooked meat products (low acidity and high a_w) and to the storage temperatures that may support the growth of the pathogens after HPP. However, results show that the conditions where *Salmonella* is able to grow after HPP in raw and cooked meat products are more restrictive than for *L. monocytogenes*. This can be attributed to the impact of storage temperature on pathogens. Considering that the minimum growth temperature for *Salmonella* is reported to be ca. 5 °C (ICMSF, 1996), refrigerated storage can be a limiting factor for the pathogen's growth. On the contrary, refrigerated storage is not a limiting factor for *L. monocytogenes* growth due to its psychotropic nature. Thereby, the recovery and subsequent growth of *L. monocytogenes* and *Salmonella* cells after HPP in raw and cooked meat products can constitute a potential risk for consumers.

In the case of dry-cured and dry-fermented meat products, results show that when HPP is applied in the final product (after curing, ripening and drying), *L. monocytogenes* and *Salmonella* are not able to grow after HPP (Table 4) due to the presence of different hurdles (e.g. low a_w, acidification, antimicrobial metabolites of lactic acid bacteria like bacteriocins, etc). In some conditions both pathogens can survive after HPP, their levels remaining constant during the subsequent storage. Some studies point out that the survival of *L. monocytogenes* after HPP in dry-cured and dry-fermented products would be favoured at refrigeration temperatures. On the other hand, a few studies explored the behaviour of *L. monocytogenes* and *Salmonella* when HPP was applied in the initial stages of the manufacturing process. The results of these studies show that both pathogens could grow after HPP if the physicochemical and storage conditions support it (Table 4).

Table 3. Studies dealing with the behaviour of *L. monocytogenes* and *Salmonella* in raw and cooked meat products after HPP

Microorganism	Product (pH/a_w)	HPP	Inoculum level before HPP (log cfu/g)	Inactivation by HPP (log N_0-N)	Behaviour after HPP (days at T°C)[c]	Reference
L. monocytogenes	Chicken fillet (5.8/-)	500 MPa, 5 min, 19 °C	6	5-5.5	**G** up to 8 log cfu/g (12d at 12°C) **S** (12 days at 4°C)	(Argyri et al., 2019)
	Chicken fillet (-/-)	300 MPa, 5 min, 15 °C	7	3.2	**S** (14d at 4°C)	(Kruk et al., 2011)
	Chicken fillet (-/-)	450 & 600 MPa, 5 min, 15 °C	7	>7[a]	**S** (14d at 4°C)	(Kruk et al., 2011)
	Cooked ham (6.1/0.98)	400 MPa, 5 min, 15 °C	4 or 7	0-4.8	**G** up to 8 log cfu/g (40d at 4°C or 20d at 8°C or 16d at 12°C)	(Hereu et al., 2014)
	Cooked ham (-/-)	400 MPa, 10 min, 17 °C	4.5	1.8	**G** up to 6.5 log cfu/g (90 days at 6°C)	(Jofré et al., 2007)
	Cooked ham (-/-)	400 MPa, 10 min, 17 °C	4	3.4 or 2.3	**S** (8d at 6°C) followed by **G** up to 8.6 log cfu/g (14d at 6°C) **I** to <QL (60 days at 1°C)	(Marcos et al., 2008)
	Cooked ham (-/-)	400 MPa, 15 min, -	3	NR[b]	**G** up to 7.5-9.9 log cfu/g (45d at 12°C or 60d at 8°C or 90d at 4°C)	(Pal et al., 2008)
	Cooked ham (6.3/0.98)	500 MPa, 10 min, 25 °C	5	>5[a]	**G** up to 7.5 log cfu/g (70d at 10°C)	(Koseki et al., 2007)
	Cooked ham (6.3/0.98)	600 MPa, 10 min, 25 °C	5	>5[a]	**<QL** (30d at 10°C) followed by **G** up to 7 log cfu/g (70d at 10°C)	(Koseki et al., 2007)
	Cooked ham (6.28/0.98)	600 MPa, 5 min, 10 °C	4	>4[a]	**<QL** (90d at 1 and 6°C)	(Jofré et al., 2008)
	Cooked ham (6.11/0.98)	600 MPa, 6 min, 31 °C	3.5	>3.5[a]	**<QL** (120d at 4°C)	(Jofré et al., 2009)
	Cooked ham (6.8/0.98)	450 MPa, 5 min, 6 °C	6	0.8	**G** up to 7.5-8 log cfu/g (35d at 4°C and 10°C)	(Montiel et al., 2015)
Salmonella	Chicken fillet (-/-)	300 MPa, 5 min, 15 °C	6	0.6	**S** (14d at 4°C)	(Kruk et al., 2011)
	Chicken fillet (-/-)	450 MPa, 5 min, 15 °C	6	3.4	**I** of 1.8 log (14d at 4°C)	(Kruk et al., 2011)
	Chicken fillet (-/-)	600 MPa, 5 min, 15 °C	6	>6 [a]	**S** (14d at 4°C)	(Kruk et al., 2011)
	Cooked ham (6.8/0.98)	450 MPa, 5 min, 6 °C	6	>6[a]	**S** (35d at 4°C) **G** up to 2.69 log cfu/g (35d at 10°C)	(Montiel et al., 2015)

[a]The concentration of pathogens immediately after HPP was below the quantification limit (QL). The inactivation of pathogens was assumed to be qualitative higher than the inoculum level but could not be quantified.
[b]NR: no reported
[c]G: growth; S: survival; I: inactivation; <QL: remained below quantification limit (QL)

Table 4. Studies dealing with the impact of HPP on the inactivation of *L. monocytogenes* and *Salmonella* and their subsequent behaviour in dry-cured and dry-fermented meat products.

Microorganism	Product (pH/a_w)	HPP	Time of inoculation/HPP	Inoculum level before HPP (log cfu/g)	Inactivation by HPP (log N_0-N)	Behaviour after HPP (days at T°C)[b]	Reference
L. monocytogenes	Dry-cured ham (5.9/0.90)	450 MPa, 10 min, 12 °C	Raw meat batter / 20h after inoculation	ca. 6	1.5	**I** of 1.5 log (60d at 4 or 8°C)	(Morales et al., 2006)
	Dry-cured ham (5.6/ 0.88)	450 MPa, 10 min, 12 °C	Raw meat batter / 20h after inoculation	ca. 6	ca. 1	**I** of 2.5 log (60d at 4 or 8°C)	(Morales et al., 2006)
	Dry-cured ham (5.9/0.92)	600 MPa, 6 min, 31 °C	Final product/ immediately after inoculation	ca. 3.5	>3.5[a]	**<QL** (120d at 4°C)	(Jofré et al., 2009)
	Dry-cured ham (5.8/0.94)	600 MPa, 5 min, 13 °C	Final product/ immediately after inoculation	ca. 1	<1[a]	**<QL** (38d at 4°C+74d at 8°C)	(Stollewerk et al., 2012)
	Dry-cured ham (5.6-5.8/0.87)	450 MPa, 10 min, 18 °C	Final product/ immediately after inoculation	6	0.55	**I** of 0.3 log (30d at 4 or 12°C)	(Pérez-Baltar et al., 2019)
	Dry-cured ham (5.9/0.92)	600 MPa, 5 min, 15 °C	Final product/ immediately after inoculation	7	ca. 4	**I** of 3.5 log (60d at 8°C)	(Hereu et al., 2012)
	Dry-cured ham (5.8/0.88)	600 MPa, 5 min, 15 °C	Final product/ Immediately after inoculation	7	ca. 2	**S** (60d at 8°C)	(Hereu et al., 2012)
	Dry-cured ham (5.9/0.864)	450 or 600 MPa, 10 min, 18 °C	Final product/ immediately after inoculation	6	0.9-1.9	**I** of 1.5-2.6 log (60d at 4 or 12°C)	(Pérez-Baltar et al., 2020)
	Dry-cured ham (6.0/0.88)	450 or 600 MPa, 10 min, 18 °C	Final product/ immediately after inoculation t	6	1.4-2.9	**I** of 0.5-0.7 log (60d at 4 or 12°C)	(Pérez-Baltar et al., 2020)
	Fermented sausage (5.5/0.80)	600 MPa, 8 min, 16 °C	Final product/ immediately after inoculation	5	3.2	**I** to <QL (30d at 4 or 18°C)	(Cava et al., 2020)
	Fuet (6.4/0.89)	600 MPa, 3 min, 19 °C	Raw meat batter/ final product	5.64	1.85	**I** of 3.4 log (30d at 20°C)	(Porto-Fett et al., 2022)
	Fuet (6.7/0.86)	600 MPa, 3 min, 19 °C	Raw meat batter/ final product	5.14	2.91	**I** to <QL (30d at 20°C)	(Porto-Fett et al., 2022)
	Fuet (5.8/0.98)	300 MPa, 10 min, 17 °C	Raw meat batter / 1d after inoculation	ca. 2.3	1	**G** of 1 log (6d at 12°C) followed by **I** of 1 log (20d at 12°C)	(Marcos et al., 2005)
	Chorizo (5.8/0.98)	300 MPa, 10 min, 17 °C	Raw meat batter / 1d after inoculation	ca. 2.3	1	**G** of 2 log (6d at 12°C) followed by **I** of 1 log (20 days at 12 °C)	(Marcos et al., 2005)
	Fermented sausage (5.7/0.88)	600 MPa, 5 min, 12 °C	Final product/ immediately after inoculation	5	0	**I** of 2.5 log (7d at 4°C + 83d at 12°C)	(Marcos et al., 2013)
	Fuet (-/-)	400 MPa, 10 min, 17 °C	Raw meat batter/ final product	6.6	0.6	**I** of 1 log (13d at 7°C) **I** of 2 log (13d at 22°C)	(Jofré et al., 2009)
	Genoa Salami (4.6/0.88)	483 or 600 MPa, 5 min, 19 °C	Raw meat batter/ final product	5.8	2.2-2.4	**I** of 0.9 log (28d at 4°C)	(Porto-Fett et al., 2010)
	Genoa Salami (4.7/0.93)	483 or 600 MPa, 5 min, 19 °C	Raw meat batter/ final product	6.1	3-3.9	**I** to <QL (28d at 4°C)	(Porto-Fett et al., 2010)

Table 4. (continuation) Studies dealing with the impact of HPP on the inactivation of *L. monocytogenes* and *Salmonella* and their subsequent behaviour in dry-cured and dry-fermented meat products.

Microorganism	Product (pH/a_w)	HPP	Time of inoculation/HPP	Inoculum level before HPP (log cfu/g)	Inactivation by HPP (log N_0-N)	Behaviour after HPP (days at T°C)[b]	Reference
Salmonella	Dry-cured ham (5.8/0.94)	600 MPa, 5 min, 13 °C	Final product/ immediately after inoculation	ca. 1	>1[a]	**<QL** (38d at 4°C+74d at 8°C)	(Stollewerk et al., 2012)
	Dry-cured ham (5.9/0.89)	400 MPa, 5 min, 12 °C	Final product/ immediately after inoculation	6.2	1.06	**I** of 3 log (60d at 8°C)	(de Alba et al., 2012)
	Dry-cured ham (5.9/0.89)	500 MPa, 5 min, 12 °C	Final product/ immediately after inoculation	6.2	2.54	**I** of 1.6 log (60d at 8°C)	(de Alba et al., 2012)
	Dry-cured ham (5.9/0.89)	600 MPa, 5 min, 12 °C	Final product/ immediately after inoculation	6.2	4.32	**I** to <QL (60d at 8°C)	(de Alba et al., 2012)
	Chorizo (5.8/0.98)	300 MPa, 10 min, 17 °C	Raw meat batter / 1 day after inoculation	ca. 2.3	0	**G** of 0.5 log (1 day at 12 °C) followed by **I** of 3 log (26 days at 12 °C)	(Marcos et al., 2005)
	Fuet (5.8/0.98)	300 MPa, 10 min, 17 °C	Raw meat batter / 1d after inoculation	ca. 2.3	0	**I** of 2.5 log (27d at 12°C)	(Marcos et al., 2005)
	Fuet (6.1/0.93)	400 MPa, 10 min, 17 °C	Raw meat batter/ final product	4	2	**I** to <QL (13d at 7°C) **I** to <QL (13d at 22°C)	(Jofré et al., 2009)
	Dry-cured ham (5.9/0.92)	600 MPa, 6 min, 31 °C	Final product/ immediately after inoculation	ca. 3.5	>3.5[a]	**<QL** (120d at 4°C)	(Jofré et al., 2009)

[a]The concentration of pathogens immediately after HPP was below the quantification limit (QL). The inactivation of pathogens was assumed to be qualitative higher than the inoculum level but could not be quantified.

[b]G: growth; S: survival; I: inactivation; <QL: remained below quantification limit (QL)

High pressure processing in combination with antimicrobials

To enhance the safety of RTE meat products, food business operators can rely on the principles of the hurdle technology concept through the intelligent combination of diverse preservative factors (hurdles/barriers) that microorganisms are not able to overcome (Leistner, 1978). In this framework, food business operators may combine the use of HPP to reduce the level of pathogens and antimicrobials to limit the subsequent growth during the product's shelf-life.

Data from literature compilation show that the efficacy of HPP in meat products formulated with antimicrobials is usually enhanced compared to that observed in meat products formulated without antimicrobials (Table 5). This would indicate that the stress and/or injury suffered by microorganisms due to the exposure to antimicrobials prior HPP, makes them more susceptible to the HPP effects. Nevertheless, literature reports that in meat products containing lactate, the hurdle technology works unexpectedly and the efficacy of HPP is reduced (Table 5). This data highlights the need to validate the simultaneous application of different intervention strategies aimed to control the hazard.

1.6. Approaches and tools to assess the microbiological safety of RTE meat products

Food business operators are required to provide evidence of the safety of their products and of the established shelf-life. This implies assessing if the foodstuff supports the growth of relevant pathogen/s and if so, quantifying the growth during the shelf-life. In the same line, the efficacy of the implemented intervention strategies has to be assessed, providing evidence that the measure will achieve the desired/requested performance criteria (CAC/GL-69, 2008).

The approaches and tools that food business operators can use to generate scientific and technical data providing evidence of the safety of their products must be selected on a case-by-case basis. The analysis and integration of all the information compiled must allow us to conclude on the adequacy (i.e. validation) of the applied intervention strategies and/or the established shelf-life. A description of procedures and tools that can be used are detailed in the next sections (Figure 6) (Ceylan et al., 2021):

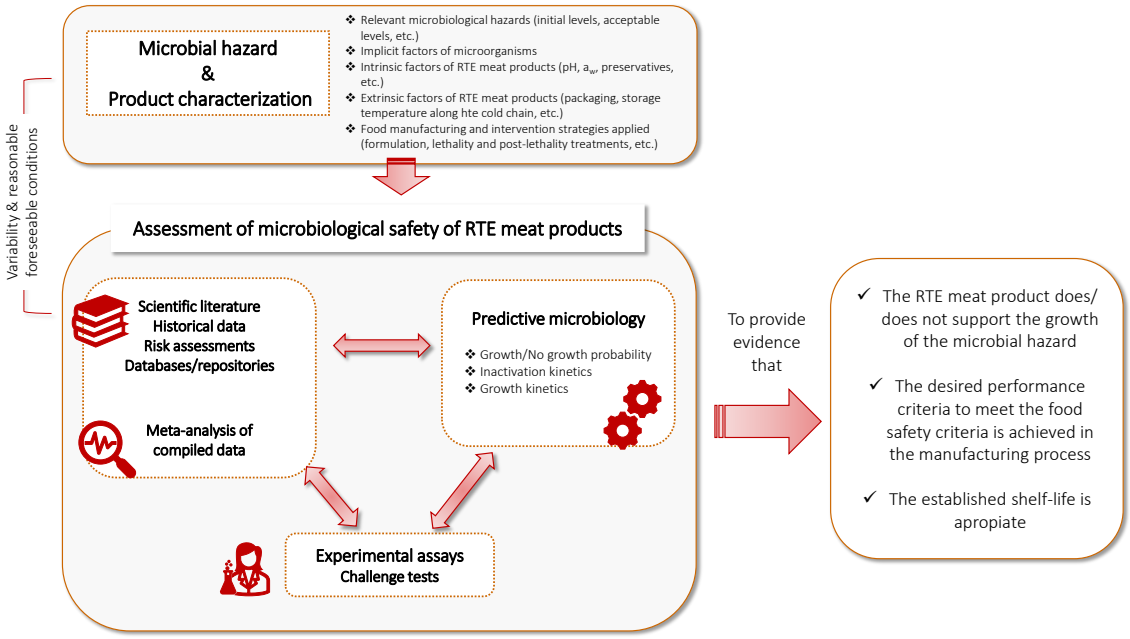

Figure 6. Approaches and tools to assess (validate) the microbiological safety of RTE meat products.

Table 5. Studies dealing with the impact of HPP on the inactivation of *L. monocytogenes* and their subsequent behaviour in RTE meats formulated with antimicrobials.

Product (pH/a_w)	Antimicrobial	HPP	Inactivation by HPP (log N_0-N)		Behaviour after HPP (days at T°C)[b]	References
			Without antimicrobials	With antimicrobials		
Chicken breast fillets (-/-)	Lactoferrin	400 MPa, 10 min, 10 °C	3.7	4.1-4.5	**G** up to 7 log cfu/g (9d at 5 °C)	(Del Olmo et al., 2012)
Chicken breast (6.1/0.95)	Coriander essential oil	500 MPa, 1 min, 8 °C	1.4	>4[a]	**<QL** (60d at 4°C) **G** up to 8 log cfu/g (60d at 8°C)	(Stratakos et al., 2015)
Cooked ham (6.8/-)	Lactoperoxidase	450 MPa, 5 min, 6 °C	0.78-1.20	3.42-3.65	**S** (35d at 4°C) **G** of 1.6 log (35d at 10°C)	(Montiel et al., 2015)
Cooked ham (6.8/-)	Reuterin	450 MPa, 5 min, 6 °C	0.78-1.20	1.79-2.60	**S** (35d at 4°C) **G** of 4 log (35d at 10°C)	(Montiel et al., 2015)
Cooked ham (-/-)	Purasal (Potassium lactate & Sodium diacetate)	400 MPa, 10 min, 17 °C	<2	>5[a]	**G** of 2 log (63d storage at 6 °C; 24-h cold chain break at 20 °C at day 40)	(Marcos et al., 2008)
Cooked ham (-/-)	Enterocins A and B	400 MPa, 10 min, 17 °C	0.15	7.79	**I** to <QL (90d at 6°C)	(Marcos et al., 2008)
Cooked ham (-/-)	Enterocins A and B	400 MPa, 10 min, 17 °C	1.09	7.87	**<QL** (90d at 1°C; 24-h cold chain break at 20°C at day 60)	(Marcos et al., 2008)
Cooked ham (-/-)	Lactate	400 MPa, 10 min	0.66	0.59	**S** (90d at 1 or 6°C)	(Aymerich et al., 2005)
Cooked ham (6.3/0.98)	Lactate	400 MPa, 10 min	1.76	1.5	**<QL** (90d at 1 or 6°C)	(Jofré et al., 2008)
Cooked ham (6.3/0.98)	Lactate	600 MPa, 5 min	3.79	3.71	**<QL** (90d at 1 or 6°C)	(Jofré et al., 2008)
Cooked turkey (6.1/0.96)	Lactate	350 MPa, 2 min	0.85	0.54	-	(Lerasle et al., 2014)
Cooked turkey (6.1/0.96)	Lactate	350 MPa, 8 min	1.42	0.81	-	(Lerasle et al., 2014)
Cooked turkey (6.1/0.96)	Lactate	350 MPa, 14 min	1.96	1.2	-	(Lerasle et al., 2014)
Dry-cured ham (5.5-5.7)	Enterocins A and B	450 MPa, 10 min, 18 °C	0.45-0.61	4.38-5.25	**S** (30d at 4 or 12°C)	(Pérez-Baltar et al., 2019)

[a]The concentration of pathogens immediately after HPP was below the quantification limit (QL). The inactivation of pathogens was assumed to be qualitative higher than the inoculum level but could not be quantified.

[b]G: growth; S: survival; I: inactivation; <QL: remained below quantification limit (QL)

1.6.1 Characterization of hazard and product

To evaluate the microbiological safety of RTE foods, it is necessary to identify the microbiological relevant hazards, i.e., microorganisms of concern that could be present in the product, characterizing implicit factors, i.e., factors related to pathogens themselves as well as other microbial groups that may interact with them and affect their behaviour (Hamad, 2012). Moreover, the food product has also to be characterized in relation to the factors (intrinsic and extrinsic factors) that can affect the microbial behaviour (Hamad, 2012). The Intrinsic factors are those properties related to the food matrix, such as pH, a_w, nutrient content or antimicrobial agents (e.g., organic acids or bacteriocins) among others. Specific intrinsic characteristics of RTE meat products may play a determinant role in allowing or not the growth of microorganisms of concern such as *L. monocytogenes* and *Salmonella*. The high a_w values and low acidification of RTE raw and cooked meat products can favour the growth of pathogens. However, the addition of antimicrobial agents into the raw and cooked meat products can limit and even suppress the growth of pathogens. On the other hand, the low a_w values of dry-cured meat products together with their acidification (in case of dry-fermented meat products) can restrict the growth of pathogenic bacteria and even favour their inactivation along the storage. In the case of the extrinsic factors, they refer to those properties/processes to which food is exposed during its manufacturing and shelf-life but that are not related to the food matrix. The main extrinsic factors found in RTE meat products are those associated with processing conditions, packaging and storage (e.g., temperature, pressure, gas composition, etc.). In the framework of RTE meat products, the number of extrinsic factors and their impact on pathogens greatly depends on the RTE meat type and its associated manufacturing process. In the case of raw meat, the main extrinsic factors are the packaging system, additional lethality strategies such as HPP and the storage temperature. On the other hand, for cooked, dry-cured, and dry-fermented meat products, extrinsic factors related to the manufacturing process must be considered in addition to the ones considered for raw meat products such as the cooking treatment conditions for cooked meat products or the relative humidity for dry-cured and dry-fermented meat products.

The characterization of the food factors allows food business operators to consider the variability of the intrinsic and extrinsic factors under reasonably foreseeable conditions between-production lots, within-production lots and within the food product. The characterization of this variability can be used to identify the worst-case scenario, i.e., scenario that greatly favours the presence and/or growth of the microorganism of concern in the food. The identification and consideration of the worst-case scenario (European Commission, 2005) is recommended by the Food and Drug Administration (FDA), the British Retail and Consortium (BRC, 2018) and other guidelines (NACMCF, 2010) when validating the efficacy of intervention strategies applied.

1.6.2 Scientific literature, historical data and meta-analysis

The collection and analysis of data dealing with the behaviour of the relevant pathogenic microorganisms in a foodstuff is very relevant, particularly when aiming to demonstrate that the food characteristics and environmental conditions do not support the growth of the microorganism of interest.

Different sources of information and data can be used including scientific literature, historical data from previous validation studies from meat industry or equipment manufacturers, risk assessments performed by organizations such as EFSA (EFSA BIOHAZ Panel, 2018) as well as specialized databases/repositories, such as *Combase (*http://www.combase.cc*)* and *Pathogens in Foods* (https://vcadavez.shinyapps.io/MeatProducts/), gathering a great number of data regarding the behaviour of pathogens under different environments.

The collection and analysis of this data can help to identify relevant factors and to quantify their impact (considering the possible associated variability) on the behaviour of the microbial hazards in the product. In this respect, the emergent use of the meta-analysis as an approach to systematically compile and analyze

the whole dataset collected from independent sources (den Besten & Zwietering, 2012). Meta-analysis can be performed using fixed effect and/or random effects statistical models. While a fixed effect assumes the same true effect size for the whole dataset obtained from different sources, a random effects considers that the true effect size of collected data could vary through different sources of data (Dettori et al., 2022).

In some instances, the collection and evaluation of data dealing with the behaviour of the relevant pathogenic microorganisms in a foodstuff can provide evidence of the ability of the foodstuff on supporting or not the growth of the target microorganism and/or providing evidence of the efficacy of the intervention strategy to control the microbiological hazard. In other instances, the extracted information resulting from the analysis of data may not cover the specific characteristics of the food mismatching with the situation of food business operators. Moreover, sometimes data covering worst-case scenarios, e.g. foreseeable conditions of the cold chain more favourable for pathogen's survival and growth is not found. Therefore, food business operators may not be able to prove the efficacy of the intervention strategies implemented and the safety of their products along the shelf-life. In those cases, the application of additional complementary approaches is needed to prove and validate the efficacy of the intervention strategies implemented as well as to set the product's safe shelf-life. These additional approaches can be based on the application of predictive microbiology tools and/or laboratory studies (challenge test or durability studies).

1.6.3 Predictive microbiology

Predictive microbiology, also called quantitative microbial ecology, is a discipline from food microbiology that aims to quantitatively characterize the behaviour of microorganisms in food as a function of environmental factors (e.g., intrinsic and extrinsic factors). Predictive microbiology relies on the principle that the microbial response is reproducible, quantifiable, and thus predictable through mathematical models. Within the scope of predictive microbiology, mathematical models describing and quantifying the microbial response (inactivation, survival, growth and/or the production of toxins) is a powerful tool for evaluating the efficacy of control measures and estimating the shelf-life of products (Pérez-Rodríguez & Valero, 2013). Predictive mathematical models can be classified according to different criteria, including kinetic vs growth boundary (growth/no growth probability) models, probabilistic vs deterministic models or empirical vs mechanistic models, among others.
In the framework of kinetic models, predictive mathematical models are frequently classified in 3 different levels according to the classification made by Whiting & Buchanan (1993).

1. *Primary models* describe the change in the number of microorganisms along time under controlled and constant (isostatic) environmental conditions and are used to estimate growth or inactivation kinetic parameters
2. *Secondary models* describe the impact of intrinsic and/or extrinsic factors on the primary kinetic parameters, and can be used for predictive purposes
3. *Tertiary models:* software tools that integrate primary and secondary models with user-friendly interfaces with the aim to facilitate non-modeler users making simulations about microbial behaviour under given conditions.

The estimation of the model parameters needs to be performed considering the goodness of fit of the mathematical model used. This can be carried out with statistical approaches allowing the comparison between the fitted values and the observed values (input data points for fitting) and calculating indexes such as the root mean square error (RMSE), which is the standard deviation of the residuals (Ratkowsky, 2004). Besides the statistical goodness of fit, the predictive performance of the developed models should ideally be validated, i.e., to compare predictions obtained with the developed model with independent

data in similar food matrices belonging to experiments not used to develop the model (Mejholm & Dalgaard, 2010).

1.6.3.1 Modelling of microbial growth

The growth curve of microorganisms is typically described in 3 different phases: lag phase, exponential growth phase and stationary phase.
Lag phase, also called adaptation time or latency time, is the period needed for microorganisms to adapt to the new environment before starting to grow. During this period, bacterial cells adapt their physiological state before they are able to start to replicate. The lag phase depends on several factors including intrinsic, extrinsic and implicit factors (Buchanan & Cygnarowicz, 1990). Moreover, the physiological state derived from any stress suffered by bacterial cells due to their exposure to some lethal or sublethal treatments such as antimicrobials, HPP can promote an extended lag time. Exponential growth phase is the period when bacterial population increases through binary fission (cell doubling). The rate of bacterial population will increase at a constant growth rate as long as there are no changes in the environment. The stationary phase is the period where the bacteria stop growing due to growth-limiting factors such as depletion of nutrients or inhibitory metabolites and it reflects the situation where the number of cell doubling is equal to the number of dying cells.

1. *Primary growth models*

Primary growth models describe microbial growth kinetics under controlled and constant environmental conditions. Primary models are based on the exponential model (Table 6).

Table 6. Primary growth models to fit the *L. monocytogenes* growth data as a function of time.

Model	Equation[a]
Exponential model	$\mathrm{Log}(N_t) = \mathrm{Log}\left(N_0 \cdot \exp(\mu_{max} \cdot t)\right)$
Logistic model	$\mathrm{Log}(N_t) = \mathrm{Log}\left(\frac{N_{max}}{1 + \left(\left(\frac{N_{max}}{N_0}\right) + 1\right) * \left(\exp(-\mu_{max} * t)\right)}\right)$
Logistic model with delay	If $t < \lambda$; $\mathrm{Log}(N_t) = \mathrm{Log}(N_0)$ If $t \geq \lambda$; $\mathrm{Log}(N_t) = \mathrm{Log}\left(\frac{N_{max}}{1+\left(\left(\frac{N_{max}}{N_0}\right)+1\right)*\left(\exp(-\mu_{max}*(t-\lambda))\right)}\right)$
Baranyi and Roberts model	$\mathrm{Log}\,(N_t) = \mathrm{Log}\,(N_0) + \frac{1}{\mu_{max}} \cdot \left[t + \frac{1}{\mu_{max}} \cdot \ln\left(\frac{\exp(-\mu_{max} \cdot t) + q_0}{1 + q_0}\right)\right] - \frac{1}{\mathrm{Log}(10)} \cdot \ln\left(1 + \frac{\exp\left(\mu_{max} \cdot \left[t + \frac{1}{\mu_{max}} \cdot \ln\left(\frac{\exp(-\mu_{max} \cdot t) + q_0}{1 + q_0}\right)\right]\right) - 1}{\exp(\mathrm{Log}(N_{max}) - \mathrm{Log}(N_0))}\right)$

Where t is the storage time; λ is the lag time, N_t is the bacterial concentration (cfu/g) at time t, N_0 is the bacterial concentration (cfu/g) at time zero, N_{max} is the maximum bacterial concentration (cfu/g), μ_{max} is the maximum specific growth rate (1/time units) and q_0 is the physiological state.

To properly describe all the phases of the microbial growth curve, some kinetic parameters have been added to the baseline exponential model, including kinetic parameters describing the lag phase of microorganisms before starting to grow (logistic model with delay) and kinetic parameters describing the stationary phase where microorganisms stop to multiply (logistic model) (McKellar & Lu, 2004). Other

primary growth models are the modified Gompertz model (Zwietering et al., 1990) or the model developed by Baranyi & Roberts (1994). These models are similar to the logistic model with delay but they have the advantage of a more mechanistic basis, allowing a more accurate description of the transition phases between the lag time and maximum growth rate and between the maximum growth rate and the maximum population density.

2. Secondary growth models

Secondary growth models describe the impact of environmental conditions on the kinetic growth parameters estimated through the primary fitting. For lag time, some secondary models have been developed. Oscar (2002) and Zwietering et al. (1994) proposed the hyperbolic model to describe the lag time as a function of temperature. On the other hand, in some cases it has been observed that lag time is proportional to the maximum growth rate. This is associated with the "work to be done" by the microorganism to adapt itself to the new environment and that the rate at which microorganisms can do this work depends on its potential maximum growth rate (Robinson et al., 1998). In these situations, the relative lag time (RLT) concept is applied (Mellefont & Ross, 2003) as secondary models for lag time that directly derive from the maximum growth rate. Alternatively, quadratic polynomials can be used to empirically describe the impact of environmental factor on lag time (Buchanan, 1990; Roberts, 1995).

In the case of growth rate, different types of secondary models have been proposed. Square root-type models were originally proposed by Ratkowsky et al. (1982) describe the quadratic relationship observed between the maximum growth rate and temperature. Polynomial-type models are another type of secondary growth models generally developed independently for the growth rate (Buchanan, 1990; Roberts, 1995). The empiric nature of polynomial models has been sometimes regarded as a disadvantage because the coefficients have no biological meaning.

Another secondary growth model is the gamma concept approach, which is based on the non-dimensionality of growth factors proposed by Zwietering et al. (1993). The advantage of the application of the gamma concept is that allows the estimation of the individual inhibition effect of each factor and their combination and interaction on the microorganism's growth rate (Eq. 2). In these models, the individual effect of each environmental factor on the growth rate can vary from 0 to 1, with 0 indicating that the growth is completely inhibited and 1 indicating that growth rate is optimal for the considered factor (Rosso et al., 1995).

$$\mu_{max} = \mu_{opt} \cdot \prod_{i=1}^{k} \gamma_X(X_i) \cdot \xi \qquad \text{Eq. 2}$$

Where μ_{max} is the maximum growth rate of the microorganism in the tested conditions (1/time), μ_{opt} is the growth rate at optimum conditions (1/time), γ is the actual growth factor, X_i is the value of the environmental factor and ξ is the interaction between factors.

Based on the gamma concept approach, Rosso et al. (1995) proposed cardinal kinetic models that describe the inhibition effect of factors such as temperature, pH or a_w on the growth rate of microorganisms as a function of the minimum, optimum and maximum values of each studied factor for the microorganism's growth (Eq. 3).

$$\gamma_X(X_i) = \begin{cases} 0 & ,if\ X_i \le X_{min} \\ \dfrac{(X_i - X_{max}) \cdot (X_i - X_{min})^n}{(X_{opt} - X_{min})^{n-1} \cdot \left((X_{opt} - X_{min}) \cdot (X_i - X_{opt}) - (X_{opt} - X_{max}) \cdot \left((n-1) \cdot X_{opt} + X_{min} - n \cdot X_i\right)\right)} & ,if\ X_{min} < X_i < X_{max} \\ 0 & ,if\ X_i \ge X_{max} \end{cases} \qquad \text{Eq. 3}$$

Where X_i is the value of the environmental factor and X_{min}, X_{opt} and X_{max} are the minimum, optimum and maximum values for growth of the microorganism of concern.

1.6.3.2 Modelling of microbial inactivation

1. Primary inactivation models

There are different primary inactivation models that describe different inactivation kinetics behaviour of a microorganism along time. Some examples are presented in Table 7.
Classically, linear relationship has been considered between the lethal factor (e.g. temperature) and the logarithm of the survivors, the slope being the inactivation rate (log linear model originally described by Bigelow & Esty (1920)). Frequently, the kinetic parameter used to describe the resistance of a microorganism for a given constant value of the lethal factor is the D-value, which is defined as the time required to reduce 90% the microbial population (i.e. 1 log reduction) and it is inversely proportional to the inactivation rate. In addition, the log-linear model with shoulder and tail can be used in cases where the inactivation of microorganisms shows either or both a shoulder (population maintaining levels during a period of time before starting to inactivate) and a resistant (population resistant to lethality) tail (Geeraerd et al., 2000).
On the other hand, the Weibull model can be used in cases where the inactivation of microorganisms is non-linear (van Boekel, 2002). The kinetics parameters used to describe the inactivation kinetics of microorganisms are δ (delta, time for the first log reduction), and p (shape of the inactivation curve). The main advantage of the Weibull model is its versatility, allowing to describe concave and convex inactivation kinetic shapes.

Table 7. Primary inactivation models used to fit the *L. monocytogenes* inactivation data as a function of time.

Model	Equation[a]
Log-linear	$\mathrm{Log}(N/N_0) = -\left(\frac{k_{max} \cdot t}{\ln(10)}\right) = \frac{t}{D}$
Log-linear with tail	$\mathrm{Log}(N/N_0) = \mathrm{Log}\left[\left(1 - 10^{\mathrm{Log}(N_{res})}\right) \cdot \exp^{(-k_{max} \cdot t)} + 10^{\mathrm{Log}(N_{res})}\right]$
Log-linear with shoulder	If $t \leq shoulder$; $Log(N/N_0) = 0$ If $t > shoulder$; $\mathrm{Log}(N/N_0) = -\left(\frac{k_{max} \cdot t}{\ln(10)}\right) + \mathrm{Log}\left(\frac{\exp^{(k_{max} \cdot shoulder)}}{1 + [\exp^{(k_{max} \cdot shoulder)} - 1] \cdot \exp^{(-k_{max} \cdot t)}}\right)$
Weibull	$\mathrm{Log}(N/N_0) = -\left(\frac{t}{\delta}\right)^p$

[a] Log (N/N_0): bacterial inactivation as log reductions at specific time (t); k_{max}: inactivation rate (1/time); t: time; D: time required to reduce 90% the microbial population; Log N_{res}: maximum inactivation, tail; *shoulder*: time before inactivation (initial resistance to stress); δ: time for the first Log reduction; p: shape of the inactivation curve.

2. Secondary inactivation models

The first secondary inactivation model developed was associated with the Bigelow model, describing the increase in temperature needed to reduce the D-value by a factor of 10 (Bigelow & Esty, 1920). This approach has also been used to quantify the impact of other environmental parameters (such as pH or a_w) on the D-value (Alvarenga et al., 2021).

Alternatively, some secondary inactivation models have been proposed based on polynomial models to characterize the impact of the environmental factors on the estimated kinetic inactivation estimated with the log-linear and Weibull model types (Ross & Dalgaard, 2004). Another approach proposed by Coroller et al. (2015) consists of the application of the gamma-like concept to estimate lambda parameters quantifying the effect of the combination of the most relevant factors and their interaction on the delta parameter, i.e., time for the first log reduction of the primary Weibull model.

1.6.3.3 Modelling of microbial competition

Competition models describe the interaction between pathogens and the endogenous microorganisms or microbial cultures added. Currently, there are different competition models developed, including the models that rely on the Jameson-effect or the ones described by Lotka-Volterra (Table 8) (Cornu et al., 2011).

Jameson-effect models are based on the hypothesis that the minor population (pathogen, microorganisms of concern) stops growing when the major population (endogenous microorganisms, cultures added) reaches its maximum, resulting in a lower maximum population density of the minor population but without affecting its lag time nor growth rate (Jameson, 1962; Ross et al., 2000). Some modifications of the Jameson-effect model have been proposed, including the Jameson-effect model with N_{cri} that describes that the minor population stops growing at a critical concentration of the major population (Le Marc et al., 2009) and the Jameson-effect with gamma that describes that the minor population can continue growing (at the same or slower rate) or inactivates after the major population reaches its maximum population density (Giménez & Dalgaard, 2004). On the other hand, the Lotka-Volterra model is a predator-prey model that describes microbial competition through assuming a linear decay in the growth rate of pathogenic microorganisms with increasing microbial populations of endogenous microorganisms or added cultures (Vereecken et al., 2000).

1.6.3.4 Software tools

Software tools integrating predictive microbiology models through user-friendly interfaces are often developed to facilitate their application by non-modeler users (Koutsoumanis et al., 2016; Possas et al., 2022; Tenenhaus-Aziza & Ellouze, 2015). Available software tools include (i) model fitting tools for fitting growth/inactivation models to experimental data and estimate kinetic parameters and/or (ii) predictive tools for predicting/simulating the growth and inactivation as a function of input factors, i.e., intrinsic and/or extrinsic factors of product.

Depending on the fit-for-purpose software functionalities, these tools can be helpful for determination of safe shelf-life and shelf-life of microbiologically perishable food, assessment of the efficacy of the intervention strategies applied in extending shelf-life of foods and/or in the fulfilment with the microbiological criteria, development of products with formulation inhibiting the growth of pathogens (safety by design) or for experimental design of laboratory assays.

Table 8. Example of competition models applied in meat products based on the existence of a minor population (*L. monocytogenes*) and major population (bioprotective culture).

Competition model	Formula	
Simple Jameson-effect	$t < \lambda_{Ls}$,	$\frac{dN_{Ls}}{dt} = 0$
	$t \geq \lambda_{Ls}$,	$\frac{dN_{Ls}}{dt} = N_{Ls} \cdot \mu_{maxLs} \cdot \left(1 - \frac{N_{Ls}}{N_{maxLs}}\right) \cdot \left(1 - \frac{N_{Lm}}{N_{maxLm}}\right)$
	$t < \lambda_{Lm}$,	$\frac{dN_{Lm}}{dt} = 0$
	$t \geq \lambda_{Lm}$,	$\frac{dN_{Lm}}{dt} = N_{Lm} \cdot \mu_{maxLm} \cdot \left(1 - \frac{N_{Lm}}{N_{maxLm}}\right) \cdot \left(1 - \frac{N_{Ls}}{N_{maxLs}}\right)$
Modified Jameson-effect with N_{cri}	$t < \lambda_{Ls}$,	$\frac{dN_{Ls}}{dt} = 0$
	$t \geq \lambda_{Ls}$,	$\frac{dN_{Ls}}{dt} = N_{Ls} \cdot \mu_{maxLs} \cdot \left(1 - \frac{N_{Ls}}{N_{maxLs}}\right) \cdot \left(1 - \frac{N_{Lm}}{N_{criLm}}\right)$
	$t < \lambda_{Lm}$,	$\frac{dN_{Lm}}{dt} = 0$
	$t \geq \lambda_{Lm}$,	$\frac{dN_{Lm}}{dt} = N_{Lm} \cdot \mu_{maxLm} \cdot \left(1 - \frac{N_{Lm}}{N_{maxLm}}\right) \cdot \left(1 - \frac{N_{Ls}}{N_{criLs}}\right)$
Modified Jameson-effect with γ	$t < \lambda_{Ls}$,	$\frac{dN_{Ls}}{dt} = 0$
	$t \geq \lambda_{Ls}$,	$\frac{dN_{Ls}}{dt} = N_{Ls} \cdot \mu_{maxLs} \cdot \left(1 - \frac{N_{Ls}}{N_{maxLs}}\right) \cdot \left(1 - \frac{N_{Lm}}{N_{maxLm}}\right)$
	$t < \lambda_{Lm}$,	$\frac{dN_{Lm}}{dt} = 0$
	$t \geq \lambda_{Lm}$,	$\frac{dN_{Lm}}{dt} = N_{Lm} \cdot \mu_{maxLm} \cdot \left(1 - \frac{N_{Lm}}{N_{maxLm}}\right) \cdot \left(1 - \frac{\gamma \cdot N_{Ls}}{N_{maxLs}}\right)$
Lotka-Volterra	$t < \lambda_{Ls}$,	$\frac{dN_{Ls}}{dt} = 0$
	$t \geq \lambda_{Ls}$,	$\frac{dN_{Ls}}{dt} = N_{Ls} \cdot \mu_{maxLs} \cdot \left(1 - \frac{N_{Ls} + F_{LsLm} \cdot N_{Lm}}{N_{maxLs}}\right)$
	$t < \lambda_{Lm}$,	$\frac{dN_{Lm}}{dt} = 0$
	$t \geq \lambda_{Lm}$,	$\frac{dN_{Lm}}{dt} = N_{Lm} \cdot \mu_{maxLm} \cdot \left(1 - \frac{N_{Lm} + F_{LmLs} \cdot N_{Ls}}{N_{maxLm}}\right)$

where for *bioprotective culture* (*Ls*) and *L. monocytogenes* (*Lm*), λ is the lag time (d), N is the bacterial concentration (Log cfu/g) at time t, μ_{max} is the maximum specific growth rate (d^{-1}), N_{max} is the maximum population density (Log cfu/g), γ is a interaction factor that allows *L. monocytogenes* to increase (γ <1) or decrease (γ >1) after the culture has reached its N_{max}, N_{cri} is the maximum critical concentration that a population should reach to inhibit the growth of the other population, F_{LsLm} and F_{LmLs} are the competition factors.

1.6.4 Experimental assays. Challenge tests

Challenge tests are experimental assays where the behaviour (inactivation or growth) of a microorganism deliberately inoculated in a product at known concentration is assessed. Challenge testing is one of the recognized approaches used to validate control measures, food production processes, food storage conditions, and food preparation recommendations for consumers aiming at microbiological safety and quality of food (CAC/GL-69, 2008). As described by the ISO standards (ISO 20976-1, 2019; ISO 20976-2, 2022) and the EURL-Lm guidelines (EURL-Lm, 2021), there are two types of challenge tests:

1. Aimed to determine the **growth potential** (log increase) or the **inactivation potential** (log decrease) of a microorganism in food, e.g., the difference between the highest or lowest concentration of

the microorganism (log cfu/g) during the test, respectively, and the initial concentration (log cfu/g). The determination of the growth potential can provide evidence about the ability of the foodstuff to support the growth of the microorganism of interest, while the inactivation potential provides data to support the validation about the achievement of the performance criteria set for the studied intervention strategy.

2. Aimed to determine the **maximum growth rate or the inactivation rate** of a microorganism in food. For that, studies must be carried out at constant (e.g., isothermal) conditions and a periodic enumeration of bacteria (between 10 and 15 points per assay) would allow to estimate the kinetic parameters through the fit of a primary predictive model to the obtained data. The determination of the growth/inactivation rate of microorganisms in food can be used to set up process conditions and to simulate different shelf-life (storage temperature) scenarios. From this, food business operators can include process and product criteria in their HACCP plan with the aim to accomplish the food safety objective.

Besides their usefulness for food business operators, challenge tests are also the way of generating data used to develop predictive models (Section 1.6.3).

The abovementioned challenge tests shall be carried out considering the inherent variability linked to the product, the variability in the target microorganism's response and the variability associated with the processing and storage conditions. For example, this would imply to identify the product and process conditions for the pathogen's survival and growth and to carry out the challenge tests with strains previously characterized to be more resistant to the conditions assayed compared to others. Moreover, to take into consideration the variability associated with the study, the use of technological and biological replicates is highly encouraged (Ceylan et al., 2021). Increasing the number of replicates the uncertainty of the results can be minimized. However, the number of biological replicates and independent trials need to be designed fit for purpose considering the variability of the system being assessed (NACMCF, 2010).

Currently, there are some publications providing guidelines for the development of challenge tests. The European Reference Laboratory developed a technical guidance document with recommendations on how to plan, execute and interpret the results from challenge tests aimed to assess the behaviour of *L. monocytogenes* along the product's shelf-life (EURL-Lm, 2021). Besides this document, the International Organization for Standardization (ISO 20976-1, 2019; ISO 20976-2, 2022), the National Advisory Committee on Microbiological Criteria for Foods (NACMCF, 2010) and several health authorities such as Ireland, United Kingdom and Canada have published guidelines for the development of challenge tests for *L. monocytogenes* (FSAI, 2022; Health Canada, 2012; HPA, 2009).

OBJECTIVES

2. Objectives

The main objective was to assess the impact of different intervention strategies to improve the safety and to extend the safe shelf-life of different types RTE meat preparations and products.
To achieve this main objective, the following specific objectives were identified:

1. To assess the behaviour of *L. monocytogenes* and *Salmonella* in RTE meat products vacuum-packed, without intervention strategies (as control baseline scenario).

2. The assess the impact of antimicrobial strategies aimed to inhibit the growth of *L. monocytogenes*, including biopreservation and modified atmosphere packaging (MAP) on the growth kinetics of the pathogen in refrigerated cooked meat products.

3. To evaluate the use of high-pressure processing (HPP) as a post-lethality strategy aimed to inactivate *L. monocytogenes* and *Salmonella* in RTE meat products.

4. To explore additive, synergistic, or antagonistic interactions of the application of HPP in combination with antimicrobial strategies, including biopreservation, MAP and/or with the implementation of a corrective storage prior commercialization.

5. To evaluate the impact of the implementation of a corrective storage to inactivate *Salmonella* and *L. monocytogenes* in RTE meat products with low a_w.

MATERIAL AND METHODS

3. Material and Methods

The behaviour of pathogens in different RTE meat products was assessed through challenge testing and mathematical modelling approaches. The design of experiments associated with the objectives for each study and the specific material and methods are detailed in the respective scientific Articles included in Section 4 (Results).
In this section, an overview of the intervention strategies, the conditions assessed and the methodological approach followed in the different studies are summarized. In addition, the methodology used to globally analyze and discuss the entire set of data and their application for meeting food safety standards are provided.

3.1. Overview

The assessment of the efficacy of different strategies to control pathogenic bacteria in RTE meat products was performed in four different RTE meat products representative of the major categories linked to notifications and/or foodborne illnesses due to the presence of *L. monocytogenes* and/or *Salmonella* (Figure 7):

1) Raw pet food: the manufacturing of raw pet food without intervention strategies does not include a lethality step. *Salmonella* was identified as the main relevant pathogen occurring in raw meat and responsible for salmonellosis cases both in pets and humans, followed by *L. monocytogenes* as a potential hazard to contaminate the product for their ability to survive in cold industrial environments.
2) Cooked ham: the manufacturing of cooked ham includes a lethality cooking step inactivating pathogenic vegetative bacteria initially present in raw meat. During the post-cooking operations preparing convenience formats, such as slicing and packaging, cooked meat products are exposed to contamination with *L. monocytogenes*. Usually, cooked meat products support the growth of the pathogen making them the main RTE food associated with listeriosis.
3) Dry-cured ham: the manufacturing of dry-cured ham includes salting and drying steps that contribute to inhibit the growth and favour the inactivation of pathogens. However, the occurrence of *Salmonella* and *L. monocytogenes* are reported in dry-cured meat products intended to be eaten raw, either due to the pathogen resistance to production process or to post-processing contamination during packaging in convenience format.
4) Dry-fermented sausages: the manufacturing of dry-fermented sausages includes fermentation and ripening/drying processes that contribute to inhibit the growth and favour the inactivation of pathogens initially present in raw meat. *Salmonella* was identified as the main relevant pathogen in whole-sausage pieces for its potential presence in raw meat and the reported resistance to fermentation/drying processes.

The study of the efficacy of the intervention strategies was performed considering its application under foreseeable industrial conditions. A summary of the evaluated strategies to inhibit the growth and/or to inactivate *L. monocytogenes* and/or *Salmonella* in RTE meat products is gathered in Table 9.
For intervention strategies aiming to inhibit the growth, the behaviour of *L. monocytogenes* was evaluated in cooked ham formulated with organic acid salts (lactate, diacetate and their combination) (Article 6), in the presence of the antilisteria bioprotective culture *Lactobacillus sakei* CTC494 (Article 9) or for different packaging systems (vacuum and modified atmosphere containing CO_2) (Article 8). In the case of organic acid salts and the bioprotective culture CTC494, assessments were performed at refrigeration

temperatures but also at temperature abuse (15 or 20 °C) to explore worst-case scenarios, e.g., conditions more favorable for *L. monocytogenes* growth.

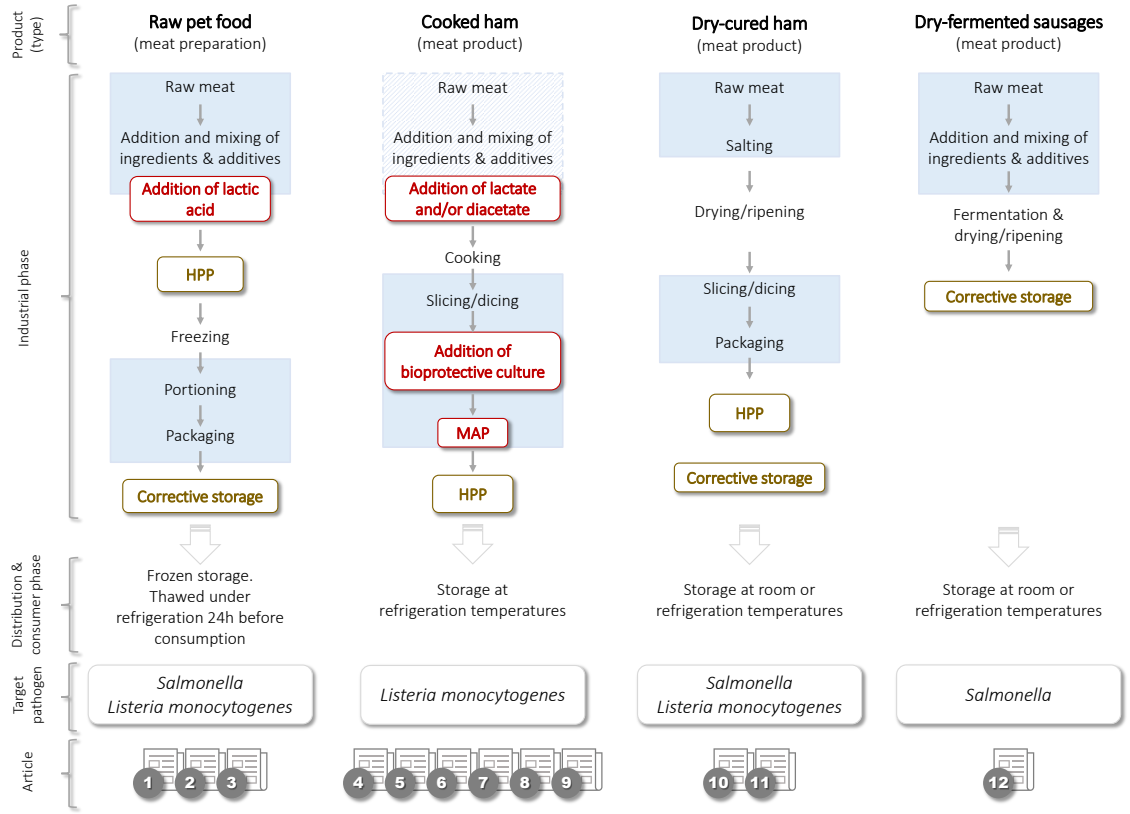

Figure 7. Summary of the intervention strategies assessed to control *L. monocytogenes* and/or *Salmonella* in different types of RTE meat products considering their processing and distribution conditions. In blue, stages of manufacturing process where pathogens can contaminate raw materials/products (dashed lines correspond to contamination eliminated by the cooking, not addressed in this PhD). In red, the assessed antimicrobial strategies aiming to limit the growth and in brown, the post-lethality strategies aiming to reduce the levels (inactivate) of pathogens and in brown target pathogens.

For intervention strategies aiming to inactivate, the behaviour of *L. monocytogenes* and *Salmonella* was evaluated in response to HPP and a corrective storage. More precisely, the efficacy of HPP was addressed in a wide variety of RTE meat products, including raw (Articles 1-3), cooked (Articles 4-8) and dry-cured (Article 10) meat products. HPP was applied in RTE meat products as in-pack post-lethality treatment, except for raw pet food, for which HPP was applied as a hygienization treatment before the final packaging and freezing. Different HPP treatments (in terms of pressure level and holding time) that, based on scientific literature and at foreseeable industrial conditions, were more suitable to inactivate the target pathogen considering the intrinsic and extrinsic factors (pH, a_w, formulation with organic acids, packaging) of the RTE meat products. Additionally, the behaviour of *L. monocytogenes* and *Salmonella* in response to HPP combined with intervention strategies aiming to limit the growth was also evaluated to explore additive, synergistic and/or antagonistic interactions in the immediate effect of the HPP and also during the subsequent storage (Articles 1-8).

In the case of corrective storage, the behaviour of *L. monocytogenes* in dry-cured ham and of *Salmonella* in dry-fermented sausages with different physicochemical characteristics (pH and a_w) and stored at refrigeration or room temperatures was evaluated (Articles 11-12).

Table 9. Summary of intervention strategies assessed in the studies included in the PhD to control. *L monocytogenes* and *Salmonella* in RTE meat foods.

Article	Pathogen[a] [strain(s)]	RTE food[b] [pH/a_w]	Intervention strategies					Purpose of the study	Methodological approaches (mathematical/statistical analysis)
			Organic acids [concentration]	Bioprotective culture	MAP[c]	HPP[d] MPa/min/°C	Corrective storage °C /days		
1	*Salmonella* [CECT702; CECT4565; CECT705; CTC1003; CTC1022; CECT34136T; CCUG21272; GN0085; GN0082; CTC1756] *L. monocytogenes* [12MOB045LM; 12MOB089LM; CTC1011; Scott A; CECT4031T; CTC1034; 12MOB102LM; CTC1769; 12MOB049LM; 12MOB050LM]	RPFD [5.7-6.8/0.991]	Lactic acid [3.6 g/kg]	-	-	400/5/9	-	To screen select strains of *Salmonella* and *L. monocytogenes* suitable for assessing HPP efficacy	Challenge test for inactivation potential (Descriptive statistics)
	Salmonella [CTC1022; GN0082; GN0085]	RPFD [5.7-6.8/0.991]	Lactic acid [3.6 g/kg]	-	-	600/0-10/9	-	To characterize HPP inactivation kinetics of *Salmonella* and the impact of sublethal injury[e]	Challenge test for inactivation kinetics (Log logistic with tail model)
2	*Salmonella* [Cocktail (CTC1022; GN0082; GN0085)]	RPFD [5.7-6.6/0.991]	Lactic acid [0-7.2 g/kg][f]	-	-	450-750/0-7/9[f]	-	To develop a decision supporting for assessing HPP and setting the HPP parameters efficacy for *Salmonella* in raw pet food for dog without or with lactic acid	Challenge test for inactivation potential (Response surface methodology)
3	*Salmonella* [Cocktail (CTC1022; GN0082; GN0085)]	RPFC [5.5-6.8/0.991]	Lactic acid [0-7.2 g/kg][f]	-	-	450-750/0-7/9[f]	-18 °C/20	To develop a decision supporting for assessing the efficacy and setting the HPP parameters and subsequent corrective storage for *Salmonella* in raw pet food for cat without or with lactic acid	Challenge test for inactivation potential (Response surface methodology)
4	*L. monocytogenes* [CTC1011; CTC1034; Scott A]	CH [6.01-6.13/ 0.971-0.978]	*Lactate [1.4 or 2.8%] *Diacetate [0.11%] *Lactate [1.4%] + Diacetate [0.11%]	-	-	400/0-10/13	-	To quantify the protective effect of lactate on *L. monocytogenes* inactivation by HPP	Challenge test for inactivation kinetics (Weibull model)
5	*L. monocytogenes* [CTC1034; EGDe]	CH medium [7.0/0.978-0.985]	Lactate [2.8%]	-	air[g]	400/10/10	-	To decipher the molecular mechanisms associated with the protective effect of lactate on *L. monocytogenes* inactivation by HPP	Transcriptomics, differential gene expression (bioinformatics)
6	*L. monocytogenes* [CTC1034; Cocktail (12MOB045LM; Scott A; 12MOB089LM)]	CH [5.9-6.2/ 0.962-0.974]	*Lactate [2.8 or 4%] *Lactate [2 or 2.8%] + Diacetate [0.45 or 0.11%]	-	-	600/3/15	-	To assess the behaviour of *L. monocytogenes* after HPP in cooked ham formulated without and with organic acids at 8-20°C	Challenge test for growth kinetics (Log logistic model + secondary polynomial models for μ_{max})
7	*L. monocytogenes* [CTC1034]	CH [6.18-6.32/ 0.975-0.978]	-	-	20% CO_2; 80% N_2	400/0-15/10	-	To assess the impact of packaging on the behaviour of *L. monocytogenes* during HPP	Challenge test for inactivation kinetics (Weibull model)

Table 9 (continuation). Summary of intervention strategies assessed in the studies included in the PhD to control *L. monocytogenes* and *Salmonella* in RTE meat foods.

Article	Pathogen[a] [strain(s)]	RTE food[b] [pH/a_w]	Intervention strategies					Purpose of the study	Methodological approaches (mathematical/statistical analysis)
			Organic acids [concentration]	Bioprotective culture	MAP[c]	HPP[d] MPa/min/°C	Corrective storage °C /days		
8	*L. monocytogenes* [CTC1034]	CH [6.18-6.32/ 0.975-0.978]	-	-	20% CO_2; 80% N_2	600/3/10	-	To assess the impact of packaging on the behaviour of *L. monocytogenes* during HPP and subsequent storage at 6°C	Challenge test for growth kinetics (Log logistic model)
9	*L. monocytogenes* [CTC1034]	CH [6.07/0.978]	-	*L. sakei* CTC494[h]	-	-	-	To assess the use of *L. sakei* CTC494 as a bioprotective culture to control *L. monocytogenes* in cooked ham stored at 2-15°C	Challenge test for growth kinetics (Modified Jameson effect model with gamma + secondary polynomial models for μ_{max} and N_{max})
10	*Salmonella* [CTC1003] *L. monocytogenes* [CTC1034]	DCH [5.7/0.870-0.980][i]	-	-	-	300-852/5/15[i]	-	To assess the impact of HPP on the behaviour of *Salmonella* and *L. monocytogenes* during HPP and subsequent storage at 7°C	Challenge test for growth/no growth boundary and inactivation/growth kinetics (Gamma approach + Weibull/ Log logistic model)
11	*L. monocytogenes* [CTC1034]	DCH [5.7-5.9/ 0.850-0.910]	-	-	-	-	2, 8, 15, 25/180	To provide a decision supporting system to assess corrective storage efficacy for *L. monocytogenes* in dry-cured ham	Challenge test for inactivation kinetics (Weibull model + secondary polynomial model for kinetic parameters)
12	*Salmonella* [Cocktail (CTC1003, CTC1022, CTC1754)]	DFS [4.6-5.3/ 0.880-0.930]	-	-	-	-	4, 8, 15, 25/ 90	To provide a decision supporting system to assess corrective storage efficacy for *Salmonella* in dry-fermented sausages	Challenge test for inactivation kinetics (Weibull model + secondary polynomial model for kinetic parameters)

[a] *L. monocytogenes* and *Salmonella* cultures grown till stationary phase at 37 °C without (Article 10) or with subsequent freezing at -80 °C (Articles 1-9, 12) or at 8 °C (Article 11) were used.
[b] RPFD: raw pet food intended for dog; RPFC: raw pet food intended for cat; CH: cooked ham; DCH: dry-cured ham; DFS: dry-fermented sausage.
[c] MAP: modified atmosphere packaging; CO_2: carbon dioxide; N_2: nitrogen. Products not packed in MAP were packed under vacuum conditions.
[d] Pressure (MPa), pressure holding time without considering the come-up time (min) and initial temperature of the pressurization fluid (°C).
[e] Samples were enumerated immediately after HPP and at 24h after HPP (kept at 4°C)
[f] Values were selected according to a Central Composite Design (CCD) with 3 factors, e.g., HPP level (450 to 750 MPa), holding time (0 to 7 min) and lactic acid (0 to 7.2 g/kg), and 5 levels.
[g] Thermosealed with air.
[h] *L. sakei* CTC494 is a bioprotective culture producing Sakacin K. The *L. monocytogenes*: *L. sakei* CTC494 ratios assessed were 1:1, 1:3 and 1:5.
[i] Values were selected according to a Central Composite Design (CCD) with 3 factors, e.g., HPP level (347 to 852 MPa), a_w (0.87 to 0.96) and fat (10 to 50%), and 5 levels. Additional values were selected according to a Full Factorial Design (FFD) with 2 variables, e.g., HPP level (300 to 750 MPa) and a_w (0.94 to 0.98).

3.2. Methodology for global discussion of results

Besides the data analysis performed in each study, additional approaches presented below were used to address the global discussion of the entire set of results obtained in the different studies.

3.2.1 Assessment of *L. monocytogenes* and *Salmonella* growth in RTE meats without intervention strategies (baseline)

To evaluate the ability of *L. monocytogenes* and *Salmonella* to growth in RTE meat products non-submitted to intervention strategies, the gamma concept (Section 1.6.3) was applied. This predictive modelling approach allowed to quantify the growth inhibition of pathogens as a function of intrinsic (pH, a_w) and extrinsic (storage temperature) factors associated with RTE meat products and predict the growth/no growth behaviour. For simulations, the gamma model structure described by Augustin et al. (2005) was used, with the same cardinal values for *L. monocytogenes* and *Salmonella* used in Article 10. To cover worst-case scenarios regarding the storage temperature, predictions were performed at 9.5 °C, which is the 95th percentile value of temperature[3] found at consumer level in Spain (Jofré et al., 2019), as recommended by the EURL-Lm (2021) for the evaluation of the growth potential of *L. monocytogenes*. To cover scenarios less favourable for the pathogen's growth, predictions were also performed at 3.9 °C, which corresponded to the minimum[3] mean temperature (Jofré et al., 2019). As the minimum growth temperature for *Salmonella* is 5.06 °C (ICMSF, 1996), the growth of this pathogen at 3.9 °C was not predicted.

3.2.2 Analysis of inactivation data of *L. monocytogenes* and *Salmonella* by HPP in RTE meat products

The statistical meta-analysis approach was used enabling the results of similar studies to be pooled in order to determine significant trends. Meta-analysis is well implemented in research areas such as health and epidemiology, and it is currently an emergent approach in the food safety research (den Besten & Zwietering, 2012).

Meta-analysis can use either fixed effect and/or random effects statistical models, the difference between the fixed and random effects model lies on the nature of the variability between studies (Riley et al., 2011; Spineli & Pandis, 2020). A fixed effect meta-analysis assumes that there is a common true effect size for all the included studies, i.e. the magnitude of the observed effect varies because of the random error inherent in each study (i.e. the source of error is only the within-study variation). On the other hand, a random effects meta-analysis considers that the true effect could vary between studies (i.e., the sources of error are both within-study and between-study variance).

To account for the heterogeneity caused by differences in experimental procedures between studies, fixed- and random-effects meta-analysis models were used to analyse the entire set of HPP inactivation results, as log reduction (log N/N_0) data. Particularly, the 1332 log reduction experimental data of *L. monocytogenes* and *Salmonella* reported in HPP studies from Articles 1-8 and 10 (Table 9) were analysed as a function of fixed and random factors gathered in Table 10.

[3] From the distribution of the mean from 24h-temperature profile recorded for 160 domestic refrigerators. The minimum mean corresponds to the value recorded at the core position.

Table 10. Fixed and random factors considered in meta-analysis models exploring the HPP inactivation effect.

Factor (units)	Type of factor	Range [min-max] or level
Fixed factors (all the levels of interest have been covered)		
Pressure (MPa)	Continuous	[300-852]
Holding time (min)[a]	Continuous	[0-15]
Temperature (°C)[b]	Continuous	[9-15]
Pathogen	Categoric	*L. monocytogenes; Salmonella*
Physiological state	Categoric	Grown at 37 °C; Grown at 37 and subsequently frozen at -80 °C
Type of product	Categoric	Raw; Cooked; Dry-cured
a_w	Continuous	[0.87-0.99]
pH	Continuous	[5.5-7.0]
Type of antimicrobial	Categoric	None; Lactic acid; Lactate; Diacetate; Lactate and Diacetate
Type and amount of antimicrobial	Categoric	None; 1.5 g/kg Lactic acid; 3.6 g/kg Lactic acid; 5.7 g/kg Lactic acid; 7.2 g/kg Lactic acid; 1.1% Lactate; 1.4% Lactate; 2.8% Lactate; 4% Lactate; 0.11% Diacetate; 1.4% Lactate and 0.11% diacetate; 2.8% Lactate and 0.11% diacetate; 2% Lactate and 0.45% diacetate
Formulation	Categoric	Standard; Sodium-reduced
Packaging system	Categoric	Air; Vacuum; MAP (1h prior HPP); MAP-exposed (24 h prior HPP)
Enumeration time	Categoric	Immediately after HPP; 24h post-HPP
Random factors (only a selection of all possible levels of a factor has been included in the analysis)		
Strain (nested with the factor *Pathogen*)	Categoric	*L. monocytogenes* strains: 12MOB045LM, 12MOB089LM, CTC1011, Scott A, CECT4031^T, CTC1034, 12MOB102LM, CTC1769, 12MOB049LM, 12MOB050LM, EGDe and Cocktail (12MOB045LM; Scott A; 12MOB089LM). *Salmonella* strains: CECT702, CECT4565, CECT705, CTC1003, CTC1022, CECT34136T, CCUG21272, GN0085, GN0082, CTC1756, Cocktail (CTC1022; GN0082; GN0085)
RTE meat product (nested with the factor *Type of product*)	Categoric	Raw pet food for dog; Raw pet food for cat; Cooked ham; Cooked ham model medium, Dry-cured ham
Study	Categoric	Article 1; Article 2; Article 3; Article 4; Article 5; Article 6; Article 7; Article 8; Article 10

[a]Period of time during which target pressure level is maintained.
[b]Initial temperature of the pressurization fluid.

Different random-effects meta-analysis models considering different fixed factors were assessed with the aim of determining significant trends that affected the efficacy of HPP to control *L. monocytogenes* and *Salmonella* in RTE meat products, including:

1. Model 1: considered all the fixed factors, except the factor *Type and amount of antimicrobial*. This model assessed the significance of technological factors, factors related to pathogen and factors of the food matrix on *L. monocytogenes* and *Salmonella* inactivation.

2. Model 2: considered all the fixed factors included in Model 1 but replacing the fixed factor *Type of antimicrobial* by *Type and amount of antimicrobial*. This model 2 assessed whether the impact of antimicrobials was only affected by the type or also by the amount of antimicrobials added in the product formulation.

3. Model 3: considered all the fixed factors included in Model 2 but constrained to the subset of data for RTE meat products with $a_w \geq 0.95$. Therefore, it assessed the significance of the factor a_w in the range of [0.95-0.99].

4. Model 4: considered all the fixed factors included in Model 2 but constrained to the subset of data for RTE meat products with $a_w \geq 0.96$. Therefore, it assessed the significance of the factor a_w in the range of [0.96-0.99].

Additionally, a fixed-effects linear model was also tested:

5. Model 5: considering the fixed factors *Pressure*, *Pathogen* and a_w for the subset of data for RTE meat products with $a_w < 0.96$, all belonged to the same study (Article 10, dry-cured ham formulated without antimicrobials, vacuum packed and pressurized at 300-750 MPa for 5 min, using the same strain for each pathogen).

3.2.3 Contribution of the intervention strategies on the compliance of performance objective and safe shelf-life extension

The contribution of the intervention strategies used as control measures to fulfil with the microbiological criteria for *L. monocytogenes* and *Salmonella* in RTE meat products was evaluated through the performance objective (PO) concept taking into account variability and/or uncertainty of the parameters (i.e., using probability distributions). For this, the approach proposed by Zwietering et al. (2010) (Eq. 4) was used:

$$H_0 - \sum R + \sum I < PO \text{ or } FSO \qquad \text{Eq. 4}$$

Where H_0 is the initial concentration of the pathogen before the application of the control measure (log cfu/g); $\sum R$ is the reduction of the pathogen level during the post-lethality treatment (log); $\sum I$ is the increase of the pathogen level e.g. during the storage and *PO* (Performance Objective) and *FSO* (Food Safety Objective) is the maximum concentration (log cfu/g) of a microbial hazard in a food at a specified step in the food chain (e.g before its release to the market or at the moment of consumption, respectively (Section 1.4).

The normal distributions characterizing the initial concentration of the pathogens in the RTE meat products before the application of post-lethality treatments used for this assessment are gathered in Table 11.

Table 11. Normal distributions describing the concentration (log cfu/g) of *L. monocytogenes* and *Salmonella* in RTE foods before the application of the intervention strategy.

Pathogen	Normal distribution[a]	Products	Source
L. monocytogenes	[0.40; 1.54]	RTE foods	Distribution calculated from the cumulative distribution reported by FDA (2003) corresponding to simulated *L. monocytogenes* concentration in contaminated 25g samples of RTE food.
Salmonella	[-1.55, 0.51]	RTE raw pet food	Distribution reported in Article 2 and calculated from the presence of *Salmonella* in production lots (including products negative and positive for *Salmonella*).
	[-2.28; 0.54]	Dry-cured and dry-fermented RTE meat products	Distribution reported by Ferrer-Bustins et al. (2021) and calculated from the presence of *Salmonella* in production lots (including products negative and positive for *Salmonella*).

[a]: [mean; standard deviation]

Zero-tolerance policies for *L. monocytogenes* and *Salmonella* require the no-detection of pathogen in 25g of product (Sections 1.4.1 and 1.4.2). Therefore, the objective (PO/FSO) for the assessment of the contribution of control measures was set up at -1.40 log cfu/g (1 cell in 25g of product).

In the particular case of *L. monocytogenes*, some microbiological criteria allow allowing a maximum of 100 cfu/g of *L. monocytogenes* along the product shelf-life. In this sense, with the aim to assess the contribution of the control measures applied on shelf-life extension, Eq. 5 was used:

$$\text{Shelf} - \text{life} = \frac{FSO + H_0 + R}{\mu_{max}/\ln(10)} - \lambda \qquad \text{Eq. 5}$$

Where H_0 is the initial concentration of the pathogen before the application of the control measure, assumed to be (-1.4 log cfu/g); $\sum R$ is the reduction of the pathogen level during the post-lethality treatment (log), λ is the lag time, μ_{max} is the *L. monocytogenes* growth rate and *FSO* is the Food Safety Objective (maximum concentration of a hazard in a food at the time of consumption that provides or contributes to the appropriate level of protection, ALOP, see section 1.4).

RESULTS

Article 1

Enhanced high hydrostatic pressure lethality in acidulated raw pet food formulations was pathogen species and strain dependent

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Enhanced high hydrostatic pressure lethality in acidulated raw pet food formulations was pathogen species and strain dependent

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Abstract

Feeding dogs and cats with raw meat-based pet food is taking relevance in the recent years. The high a_w of these products together with the no cooking before its consumption by the animal pose a risk due to the potential occurrence and growth of foodborne pathogens. High pressure processing (HPP) is a non-thermal emerging technology that can be used as a lethality treatment to inactivate microorganisms with a minimum impact on the sensory and nutritional traits of the product. The purpose of the present study was to evaluate the variability in pressure resistance of different strains of the relevant foodborne pathogens *Salmonella* spp., *Escherichia coli* and *Listeria monocytogenes* in raw pet food formulated without and with lactic acid. In general, *Salmonella* and *L. monocytogenes* strains showed a higher resistance to HPP than *E. coli* strains. In lactic acid acidulated formulations, the susceptibility to HPP of *L. monocytogenes* was markedly enhanced. The resistance to HPP was not only dependent on the microorganism but also on the strain. Thus, the selection of the proper strains should be taken into account when designing and validating the application of HPP as a control measure within the HACCP plan.

Keywords:

high hydrostatic pressure, mathematical modelling, inactivation kinetics, pet food, pathogenic bacteria, piezo-resistance

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1 Introduction

Health benefit claims have been boosting pet owners to shift from traditional dry and canned pet foods to raw pet food diets (Davies et al., 2019). These types of diets are perceived as more nutritious and natural as the components are not heated and maintain the thermosensitive components, which are associated with a series of potential benefits including improved behaviour, shinier coat, better palatability and prevention of disorders affecting body systems (Davies et al., 2019; Freeman et al., 2013). In this respect, for instance, a recent observational study found significantly lower allergy/atopy skin signs after the age of 1 year in dogs eating more than 20% of diet as raw (Hemida et al., 2021).

However, the lack of heat treatments as a microbial kill step in the manufacturing process of raw pet food may pose a health risk as raw materials may harbour pathogenic bacteria (Jones et al., 2019; Nüesch-Inderbinen et al., 2019). The prevalence of bacterial pathogens has been investigated in raw pet foods. In a study conducted with 196 frozen raw pet food samples ordered online in the USA, 16.3 %, 7.6% and 4.1 % were positive for *Listeria monocytogenes, Salmonella*, and *Escherichia coli*, respectively (Nemser et al., 2014). In investigations of foodborne illnesses associated with these three pathogens in cats and dogs, raw pet food was confirmed as the incriminated food by whole genome sequencing (Jones et al., 2019). Moreover, FDA has been recalling raw pet foods contaminated with *Salmonella*, *L. monocytogenes* and *E. coli* (FDA, 2021). Due to low infectious dose, a "zero tolerance" policy for *Salmonella* in raw pet foods is implemented in many countries, e.g. in the European Union through Regulation (EC) No 142/2011 (Commission, 2011) and in the USA Compliance Policy Guide Sec 690.800 *Salmonella* in Food for Animals (FDA, 2013).

To ensure the compliance of regulatory requirements and guarantee the microbiological safety of raw pet food non-thermal preservation strategies can be applied to kill pathogenic bacteria while maintaining the nutritional and freshness traits. In this respect, High Pressure Processing (HPP) has been increasingly adopted by food and pet food producers worldwide as a killing step (Anonymous, 2019). The efficacy of HPP as microbial lethal treatment depends on the type of microorganism and the process parameters, mainly pressure and holding time (Bover-Cid et al., 2012, 2011). The physico-chemical characteristics of the food matrix have also a very strong influence on the microbial inactivation associated with HPP. Therefore, the industrial application of HPP technology needs to be validated and, whenever possible, optimised taking into account the specific pet food formulation. Moreover, pet food acidification by means of organic acids such as lactic acid has shown to be effective for inactivating *Salmonella* in rendered chicken used for raw pet food manufacture (Dhakal et al., 2019). To date, the effects of the combination of HPP technology application followed by freezing storage, currently recommended by manufacturers, with other hurdles such as acidification, on pet food microbiological safety have not been evaluated.

Studies to investigate the pressure-resistance of different strains of *Salmonella* (Sherry et al., 2004; Tamber, 2018; Whitney et al., 2007), *E. coli* (Liu et al., 2015; Whitney et al., 2007) and *L. monocytogenes* (Van Boeijen et al., 2008) in liquid culture media or phosphate buffer solution have indicated that microbial responses to HPP are diverse. Screening tests are necessary to establish the levels of pressure-resistance of different microorganisms and within the same species of a microorganism (Tamber, 2018; Van Boeijen et al., 2008). Moreover, since the pressure-resistance of a microorganism also depends on the matrix characteristics including pH and fat content (Bover-Cid et al., 2017; Li and Farid, 2016; Possas et al., 2017), the characterization of bacterial pressure-resistance in the matrix in which the implementation of HPP must be optimized or evaluated is highly recommended. Strains with the greatest resistance at different conditions should be used in challenge tests for simulating the worst-case scenarios in risk assessments (Tamber, 2018; Serra-Castelló et al., 2021).

In this framework, this work aimed at i) determining the pressure-resistance of different *Salmonella*, *E. coli* and *Listeria monocytogenes* strains in both non-acidulated and acidulated raw pet food and ii) to study the inactivation kinetics of the most pressure-resistant *Salmonella* strains in raw pet food.

2 Material & Methods

2.1 Bacterial strain and culture preparation

Individual pure cultures of the selected strains were prepared by growing a loopful of the frozen stock culture (- 80 °C) on Plate Count Agar (PCA, Merck, Darmstadt, Germany) at 37 °C overnight (18 h).

Table 1. Bacterial strains used in the present study.

Pathogen	Strain	Serotype	Origin
Salmonella enterica	CECT702	Panama (9,12:1,v:1,5)	Sewage, Albufera Lake
	CECT4565	Senftenberg (1,3,19:g,s,t)	Clinical
	CECT705	Agona (1,4,12:f,g,s:-)	Eggs
	CTC1003	London (3, 10 : l, v: 1, 6)	Pork meat
	CTC1022	Derby (1, 4, 12: f, g: -)	Pork meat
	CECT34136^T	Enteritidis (1, 9, 12:g, m:-)	Clinical
	CCUG21272	Mbandaka	Clinical
	GN0085	Typhimurium (1,4,5,12:i:1,2)	Chicken meat
	GN0082	Enteritidis (9,12:g,m:-)	Chicken meat
	CTC1756 (monophasic)	Derby (4:g,f:-)	Pork meat sausage
Escherichia coli	CTC1028	O6	Pork meat
	CTC1029	O2	Pork meat
	CTC1030	O78	Pork meat
	LMG2092^T	O1:K1:H7	Urine
	CECT5947	O157:H7 (non toxigenic; stx2-)	Human
Listeria monocytogenes	12MOB045LM	1/2c	Pork meat
	12MOB089LM	4b	Bacon
	CTC1011	1/2c	Meat
	Scott A (CCUG32843)	4b	Clinical
	CECT4031^T	1a	Meat
	CTC1034	4b	Cured ham
	12MOB102LM	4b	Salmon
	CTC1769	1/2a	Salmon
	12MOB049LM	1/2b	Industrial environment
	12MOB050LM	4b	Industrial environment

A colony was picked and grown in a new plate of PCA at 37 °C for a second overnight. Bacterial biomass was collected and resuspended with a cryoprotectant solution (0.3% of beef extract (Difco Laboratories, Detroit, MI, USA), 0.5% of Tryptone (Oxoid Ltd., Basingtok, Hampshire, UK) and 20% of glycerol) and properly distributed in aliquots. Culture was frozen at -80 °C until being used to obtain freeze-stressed cells. The frozen culture is representative of the status of the strain in raw materials usually stored frozen to produce the raw pet food.

2.2 Raw pet food preparation

The composition of a food in terms of ingredients and additives, and particularly the physico-chemical characteristics, is known to influence the efficacy of HPP. To overcome this point, the study was performed through a product-oriented approach, using the real food matrix. The raw ingredients for pet food manufacture were provided by Affinity Petcare SA (L'Hospitalet de Llobregat, Spain). The formulation of the pet food was as follows (% w/w on wet basis): chicken (80%), vegetables (18%), antioxidants (1%) and vitamins and minerals (1%). Prepared raw pet food was kept frozen at -20 °C until use.
Immediately before the experiments, the necessary aliquots of raw pet food were thawed at room temperature for 1h . For the acidulated samples, 5 ml of lactic acid based acidulant provided by Corbion® (Amsterdam, The Netherlands) (71 % v/v of lactic acid) per kg of product, was added to samples (with an initial pH of *ca*. 6.8) 24 hours before the pressurization in order to lower the pH to reach a stable pH of *ca*. 5.70. The addition of the acidulant did not significantly affect ($p > 0.05$) the a_w of the acidulated samples (a_w=0.991 ± 0.001) with respect to samples without acidulant (a_w=0.992 ± 0.001). Just before the pressurization, samples were independently inoculated with *Salmonella*, *E. coli* or *L. monocytogenes* strains at *ca*. 7.5 log cfu/g (1% v/w). Samples of 25 g were vacuum-packed in PA/PE plastic bags (oxygen permeability of 50 cm^3/m^2/24 h and a low water vapor permeability of 2.8 g/m^2/24 h; Sistemvac, Estudi Graf S.A., Girona, Spain). Samples were kept at 8 ± 1°C until being pressurized. The a_w and pH of samples were measured before and after HPP treatments with an Aqualab™ equipment (Series 3, Decagon Devices Inc., Pullman, WA, USA) and a pH meter PH25 (Crison Instruments S.A., Alella, Spain), respectively.

2.3 High pressure processing

In order to be able to quantitatively screen the pressure-resistance of different strains of *Salmonella*, *E. coli* and *L. monocytogenes* strains, a the lower pressure levels within the range of HPP usually applied at industrial level was selected. Thus, vacuum-packed raw pet food samples were pressurised at 400 MPa for a holding time of 5 min in a 120-liter Wave 6000 industrial equipment (Hiperbaric, Burgos, Spain). The pressurization fluid was water and was set up with an initial temperature of 9 °C. Compression heating was expected to be about 3°C / 100 MPa (Patazca et al., 2007). The average pressure come up rate was 200 MPa/min, while the release was almost immediate (< 6s).

2.4 Inactivation kinetics

For three *Salmonella* strains (CTC1022, GN0082 and GN0085) the kinetics of inactivation was assessed at 600 MPa, being a pressure level widely used at industrial level to increase food safety of meat products. Holding times of 0, 1, 2, 3, 5, 7 and 10 min were evaluated using the same procedures and equipment described in sections 2.1-2.3. Before microbiological analysis, pressurized samples were kept at 4 °C for 1 h. In addition, samples were microbiologically analysed after a storage of 24 hours at 4 °C in order to evaluate the potential recovery of pressure-injured cells.

2.5 Microbiological determinations

Raw pet food samples were ten-fold diluted in 0.1 % Bacto Peptone (Difco Laboratories, Detroit, MI, USA) with 0.85 % NaCl (Merck, Darmstadt, Germany) and homogenized for 60 seconds in a Smasher blender (bioMérieux, Marcy-l'Étoile, France). The homogenates were serially diluted and plated onto chromogenic media: CHROMagar™ *Salmonella* Plus (SPCM, CHROMagar, Paris, France) incubated at 37 °C for 2-5 days for the enumeration of *Salmonella*, CHROMagar Listeria (CHROMagar) incubated at 37 °C for 2-5 days for the enumeration of *L. monocytogenes* and REBECCA® EB agar (bioMérieux, Marcy-l'Étoile, France) incubated at 37 °C for 24 hours for the enumeration of *E. coli*. For samples with expected concentration of *Salmonella* or *L. monocytogenes* below the limit of detection by plate counting (4 cfu/g, resulting from

plating 4 ml of homogenate in a 14 cm diameter plate), the presence of the pathogen was investigated by enrichment of 25 g-samples 1/10 diluted and homogenized in peptone water. The presence of *Salmonella* was determined after an enrichment of the homogenate in Rappaport-Vassiliadis (RV) broth (Oxoid Ltd., Basingstoke, Hampshire, UK) for 48 h at 41.5 °C. The presence of the pathogens in the enriched homogenates was confirmed by PCR using the PrepSEQ™ Rapid Spin Sample Preparation Kit (Applied Biosystems) and MicroSEQ™ *Salmonella* spp. Detection Kit and MicroSEQ® *Listeria monocytogenes* Detection Kit (Applied Biosystems) following the instructions of the manufacturer. Microbiological determinations were conducted in pressurized and non-pressured samples in triplicate.
Inactivation of the pathogens *Salmonella* spp., *L. monocytogenes* and *E. coli* in pet food samples was expressed in terms of logarithmic reductions as the difference between counts before HPP treatments (N_0) and after treatments (N), *i.e.*, log (N_0/N).

2.6 Data analysis and curve fitting

The Log-linear with tail (Eq. 1, Geeraerd et al. (2000)) model was fitted to the *Salmonella* spp. concentration versus pressure holding time (min) data for both acidulated and non-acidulated pet food products. Data obtained immediately after HPP treatments and 24 hours after treatments were used for model fitting by using the nls2 and nls R packages (R Core Team, 2019). The root mean square error (RMSE) was calculated as measure for goodness-of-fit.
If $t \leq t_{shift}$

$$log\ (N) = log\ (N)_i - \frac{k_{max} \cdot t}{Ln(10)}$$

If $t \geq t_{shift}$

$$log\ (\mathrm{N}) = \log(N_{res})$$

Where: $log\ (N)$ bacterial concentration (log cfu/g) at a specific time (t); $log(N)_i$ is the initial bacterial concentration (log cfu/g); k_{max} is the inactivation rate (ln/min); t_{shift} is the time (min) for the appearance of resistance tail and $log\ (N_{res})$ is the residual bacterial concentration (log cfu/g).

3 Results and Discussion

3.1 HPP resistance of Salmonella spp., E. coli and L. monocytogenes in raw pet food

The results of the log reduction of the *Salmonella* spp., *E. coli* and *L. monocytogenes* strains due to HPP in products without the addition of acidulant (non-acidulated) and acidulated are shown in Figure 1.
In non-acidulated products little variability was found either between replicates within the same trial and between results from different trials and strains of *Salmonella*, *E. coli* and *L. monocytogenes*, (Coefficients of variation from 0.27 to 3.53 %). For the three pathogens studied, the strain specific resistance to HPP varied significantly ($p < 0.05$). For *Salmonella* spp., the CECT34136^T (type strain) was the most sensitive to HPP achieving 3.90 log reductions (Figure 1a). In contrast, *Salmonella* strains CTC1022, GN0085, GN0082, CTC1003 and CCUG21272 showed greater resistance (Figure 1a), with an average of logarithmic reductions of less than 0.5 log, which would not be considered microbiologically relevant considering the accuracy of the plate count determination (CAC/GL 61, 2007). Results of the present work showed that both the most sensitive strain of *Salmonella* (CECT34136^T) and one of the most resistant strains (GN0082) to HPP belonged to the Enteritidis serotype, pointing out the wide variability that can be present not only between serotypes but also between strains from the same serotype.

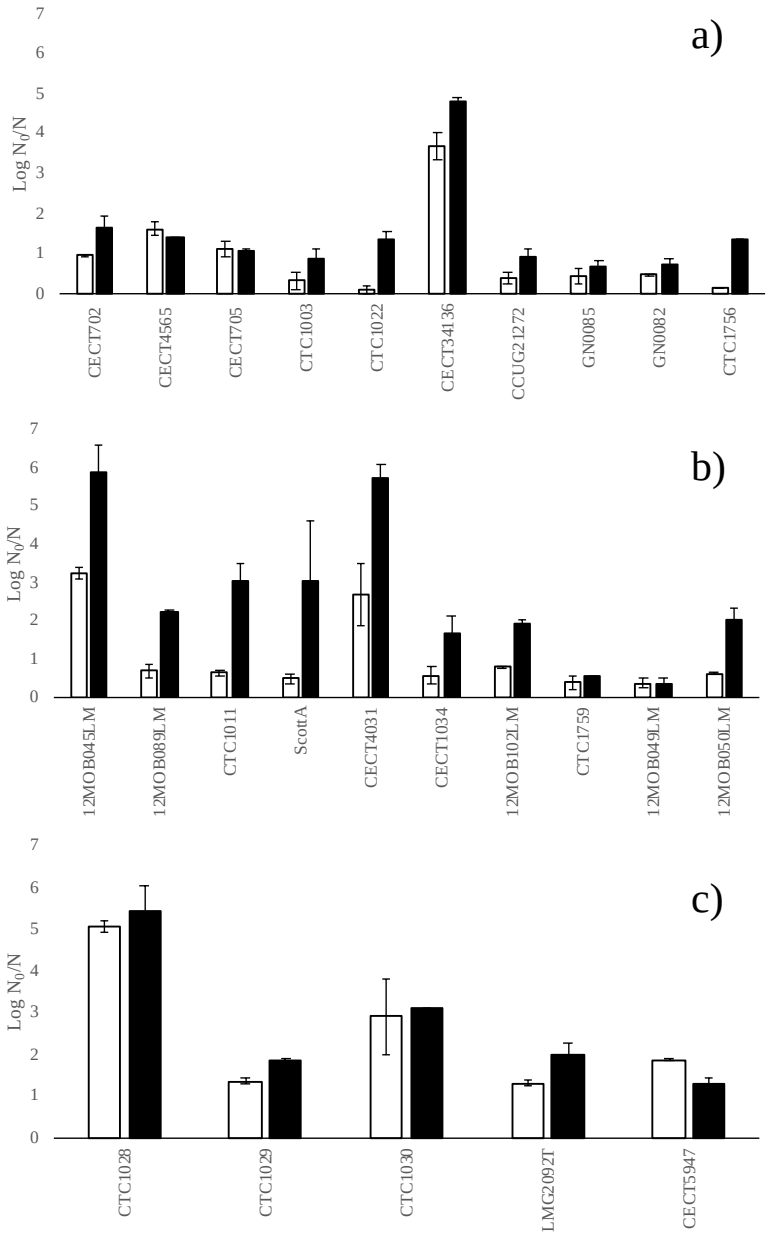

Figure 1. Mean logarithmic reductions for each strain of *Salmonella* (a), *L. monocytogenes* (b) and *E. coli* (c) strains in HPP treated (400 MPa, 5 min) raw pet food without (white bars) and with lactic acid (black bars). Standard deviation is shown with error bars.

In case of *L. monocytogenes*, the inactivation of the evaluated strains when HPP was applied in non-acidulated product was similar to that of *Salmonella* spp., being the 12MOB045LM the strain with a greatest HPP sensitivity (3.35-Log inactivation) and 12MOB049LM, CTC1769 and the clinical isolate Scott A (CCUG32843), the strains with a greatest resistance to HPP (inactivation <0.5 log) (Figure 1b). As observed for *Salmonella*, different susceptibility to HPP was found for *L. monocytogenes* strains with the same serotype (e.g. CTC1011 and EUR045LM), confirming that inactivation was more dependent on the *L. monocytogenes* strain rather than on the serotype. Comparing the three evaluated species, *E. coli* was the most sensitive to HPP (mean inactivation of 2.50 log), showing the largest variability in its inactivation response compared to *Salmonella* and *L. monocytogenes* (Figure 2), being *E. coli* CTC1029 and LMG2092^{T} the most-pressure resistant strains (1.27-1.14 log reductions) and CTC1028 the most susceptible strain (5.15 log reductions) (Figure 1c).

Generally, Gram-positive bacteria have been described as being more resistant to pressure than Gram-negative bacteria (Arroyo et al., 1997; Fonberg-Broczek et al., 2005; Moreirinha et al., 2016; Wuytack et al., 2002). However, some studies have shown that Gram-negative bacteria (especially *E. coli*) are more resistant to pressure than Gram-positive bacteria in raw poultry meats (Kruk et al., 2011; 2014; Yuste et al.,

2006). The discrepancy among the studies can be explained by the fact that the ability of microorganisms to withstand environmental stresses (not only pressure but also other food processing treatments) is much related to each specific strain rather than the characteristics of the cell envelop of Gram-positive or Gram-negative bacteria(Bartlett, 2002; den Besten et al., 2018; Considine et al., 2011Jofré et al., 2010;). In this line, in the present study, no significant differences ($p < 0.05$) in the HPP-inactivation were found between *Salmonella* and *L. monocytogenes* (Figure 2)).Moreover, although *E. coli* showed the greatest susceptibility to HPP (Figure 2), the *E. coli* strains CTC1029 and LMG2092^T showed to be more HPP-resistant than some strains of *Salmonella* (CECT34136^T) and *L. monocytogenes* (12MOB045LM and CECT4031^T) (Figure 1).
In acidulated raw pet food, and as observed in non-acidulated products, the magnitude of the HPP-inactivation of the pathogens was species and strain-dependent (Figure 1 and 2). While in *Salmonella* and *E. coli* the effect of the acidulation only resulted in a slight increase (up to *ca*. 1 log unit) of the reduction produced by HPP, the impact of acidulation was more remarkable for *L. monocytogenes*, resulting in a larger enhancement of both the lethality (up to *ca*. 3 log units more than in non-acidulated pet food) and the variability (Figure 2). It is worth mentioning that among the Gram-negative species the HPP-lethality enhancement due to lactic acid addition was not observed in a higher proportion of the strains (40 and 60% for *Salmonella* and *E. coli*, respectively), compared to *L. monocytogenes* (20%).

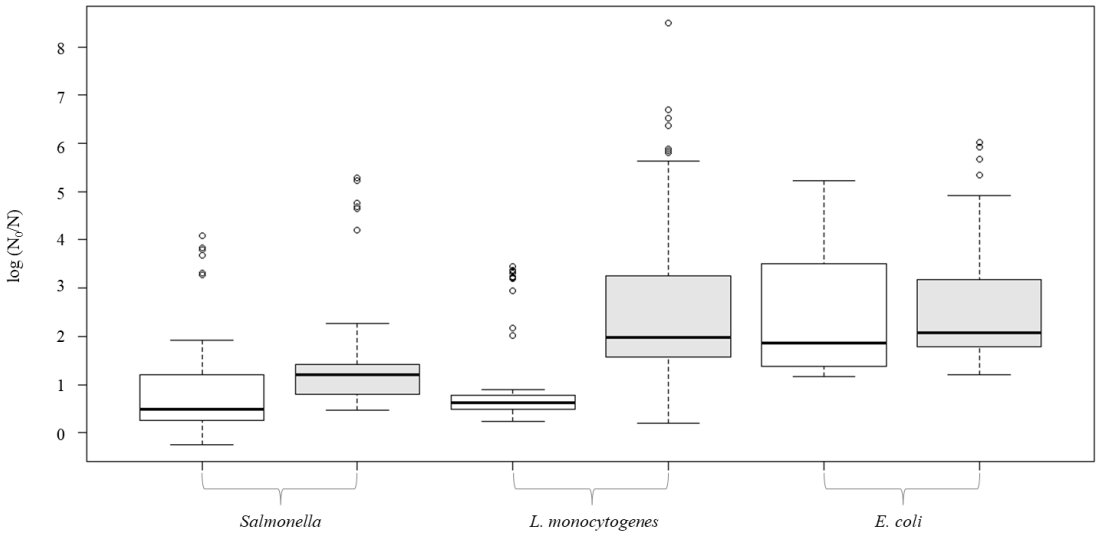

Figure 2. Boxplot of the mean log reductions of *Salmonella, L. monocytogenes* and *E. coli* strains in HPP treated (400 MPa for 5 min) raw pet food without (white boxes) and with lactic acid (grey boxes). Standard deviation is shown with error bars. Outliers are shown as empty circles.

It is well reported that one of the main consequences of HPP application on microbial cells is the membrane damage (Bowman et al., 2008). The level of the membrane damage depends on the pressure applied, being estimated that pressures at or above 400 MPa result in membrane disruption and cell leakage (Tauscher, 1995). It also depends on the membrane properties such as membrane fluidity and fatty acid composition (Casadei et al., 2002; Serra-Castelló et al., 2021). Within this context, some studies have reported that for some strains, a higher HPP-resistance of the cells is related with a larger proportion of cyclopropane fatty acids in the membrane (Charoenwong et al., 2011; Tamber, 2018). Additionally, a synergistic protective effect of cyclopropane fatty acids was reported by (Chen and Gänzle, 2016), showing that the disruption of the cyclopropane fatty acid synthase not only increased the *E. coli* lethality of the HPP treatment but also increased the *E. coli* susceptibility to lactic acid, demonstrating that this enzyme contributes to the resistance of both stresses.
Interestingly, results of the present work showed that some strains with higher sensitivity to HPP (e.g., *Salmonella* strain CECT34136T, and *L. monocytogenes* strains 12MOB045LM and CECT4031) were also more susceptible to the lactic acid addition. The same was seen for some of the most HPP-resistant or

piezo-resistant strains in which the effect of lactic acid on pathogen inactivation was less pronounced (< 0.5 log reduction difference), indicating that the presence of lactic acid practically did not modify their resistance to HPP. However, this trend was not observed for all the strains, indicating that in addition to the bacterial membrane composition, other factors may be related to the microbial resistance to HPP and acidity, such as proteins and energy-dependent cofactors. Within this framework, many proteins involved in pressure-resistance were reported to be stress proteins that their expression was governed by stress-responsive alternative sigma factors, such as σ^S and therefore, by the RpoS gene (Gayán et al., 2019, 2017; Landini et al., 2014). Additionally, (Tamber, 2018) found that differences between *Salmonella* strains resistance and their catalase activity when exposed to citric acid, suggesting a role for RpoS in coordinating the acid-resistance response and indicating that RpoS could be an important factor not only for the resistance to HPP but also for the acidity and the synergistic effect of both stresses.
Despite the intrinsic characteristics of the strains described above, the conditions in which the strains were stored before being applied to the raw food and the composition of the pet food matrix could also have had an impact on the HPP-resistance of the pathogens. As frozen raw materials are usually used for the manufacturing of raw pet food, frozen bacterial cultures were used in the present study in order to reproduce the conditions to which the pathogens could have been submitted if they were present in the raw materials. Accordingly, the results of the present study integrated the possible effect of the mechanisms developed by cells as a response to freeze stress on the resistance to subsequent stresses, e.g., HPP and acidification (Hereu et al., 2014), whose resistance will be in turn affected by the nature of the raw pet food components, as it may contain substances that affect the susceptibility of the pathogen to HPP.

3.2 Inactivation kinetics of Salmonella spp. in raw pet food by HPP and lactic acid

Since the *Salmonella* strain CTC1022 was the most pressure-resistant during screening conducted with inoculated raw pet food without lactic acid and *Salmonella* GN0082 and GN0085 showed to be the most pressure-resistant strains in raw pet food with lactic acid, their inactivation kinetics during HPP were quantitatively assessed in order to quantify the impact of HPP technological parameters (pressure level and holding time) on *Salmonella* inactivation. Data and inactivation kinetics of the 3 piezo-resistant strains of *Salmonella* (CTC1022, GN0082 and GN0085) in raw pet food treated by HPP, formulated without and with lactic acid (non-acidulated and acidulated) and enumerated immediately and 24 h after application of HPP are shown in Figure 3, and the fitted kinetic parameters of the Log-linear with tail model (Eq. 1) are summarized in Table 2.

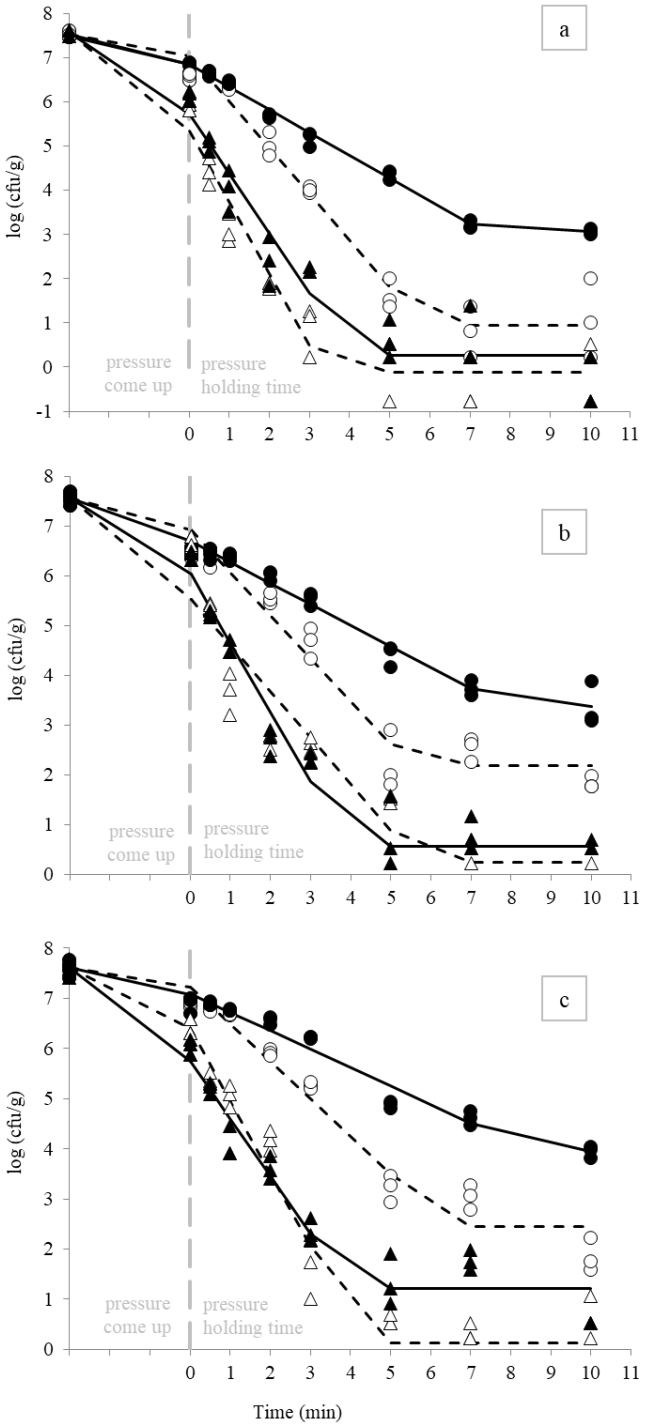

Figure 3. Inactivation kinetics of the *Salmonella* strains CTC1022 (a), GN0082 (b) and GN0085 (c) HPP treated at 600 MPa in raw pet food without (circles) and with lactic acid (triangles). Symbols represent the observed *Salmonella* counts and lines the fit of the Log-linear with tail model to data. Empty symbols and dashed lines correspond to determinations immediately post-HPP and full symbols and continuous lines correspond to determinations from 24 hours post-HPP.

Results of the enumeration performed 24 hours post-HPP resulted in a lower inactivation of *Salmonella* (between 1 to 2 Log) in products without lactic acid (non-acidulated) and in a *ca*. 1 Log in acidulated products. These results indicated that *Salmonella* could recover from sublethal injury during the storage of samples for 24 hours under refrigeration at 4 °C and be quantified by plate count in the selective chromogenic media. The differences cannot be related to growth as the minimum growth temperature of *Salmonella* is 5 - 8 °C (FSAI, 2019; (ICMSF, 1996). Recovery of the leakage of ions (Na^+, K^+, Mg^{2+} and Ca^{2+}) induced by HPP at 400 MPa has been reported in *E. coli* during subsequent storage at 20 – 37 °C for up to 24 hours (Ma et al., 2019). Although in the present study the storage temperature was lower, similar

physiological mechanisms could be employed by *Salmonella*. Several studies have reported the occurrence of sublethal damage in foodborne pathogens after HPP (Schottroff et al., 2018). From the practical point of view, these findings indicate that when the efficacy of HPP is assessed by measuring the pathogen counts immediately after the treatment, it can be overestimated.

The Log-linear with tail model clearly fitted the shape of the inactivation curve of *Salmonella* (Figure 3), indicating the presence of subpopulations with different resistance to pressure (tail effect), phenomenon that is usually reported in bacterial HPP-inactivation kinetics (Bover-Cid et al., 2012, 2011; Patterson, 2005; Patterson et al., 1995). In this context, (Ma et al., 2019) indicated that the *E. coli* cell death increased with increasing pressure (100 - 500 MPa) although the lethal effect was inconsistent with the injury effect, results that could be possibly related with the resistance tail effect.

Table 2. Estimated kinetic inactivation parameters and goodness-of-fit resulting from fitting the Log-linear with tail model to *Salmonella* inactivation data on raw pet food pressurized at 600 MPa for up to 10 min.

Strain	Product and determination time		Kinetic parameters[a]			$log\ N_{res}$ (log cfu/g)	$RMSE$
			$log(N)_i$ (log cfu/g)	k_{max} (1/min)	t_{shift} (min)		
CTC1022	Control	Immediately post-HPP	7.06 ± 0.15	2.42 ± 0.14	5.82 ± 0.29	0.94	0.440
		24 hours post-HPP	6.86 ± 0.04	1.19 ± 0.03	7.33 ± 0.18	3.06	0.124
	With lactic acid	Immediately post-HPP	5.36 ± 0.24	3.74 ± 0.33	3.37 ± 0.24	0.86	0.602
		24 hours post-HPP	5.75 ± 0.26	3.14 ± 0.35	4.03 ± 0.36	0.19	0.644
GN0082	Control	Immediately post-HPP	6.94 ± 0.15	2.00 ± 0.14	5.49 ± 0.34	2.19	0.444
		24 hours post-HPP	6.71 ± 0.07	0.98 ± 0.05	7.82 ± 0.39	3.38	0.220
	With lactic acid	Immediately post-HPP	5.55 ± 0.28	2.15 ± 0.25	5.69 ± 0.60	0.50	0.807
		24 hours post-HPP	6.06 ± 0.21	3.22 ± 0.29	3.92 ± 0.29	-0.44	0.535
GN0085	Control	Immediately post-HPP	7.24 ± 0.15	1.73 ± 0.13	6.40 ± 0.43	2.45	0.425
		24 hours post-HPP	7.09 ± 0.07	0.85 ± 0.05	8.54 ± 0.49	3.95	0.223
	With lactic acid	Immediately post-HPP	6.42 ± 0.32	3.35 ± 0.44	4.32 ± 0.46	0.14	0.813
		24 hours post-HPP	5.77 ± 0.19	2.67 ± 0.26	3.93 ± 0.31	1.21	0.482

$log(N_0/N)_i$: initial bacterial concentration; k_{max}: inactivation rate; t: time; t_{shift}: time for the appearance of resistance tail, $log(N_{res})$: residual bacterial concentration and *RMSE*: root mean square error.

[a]: Parameter estimate ± standard error

In non-acidulated products, the inactivation rate (k_{max}) estimated immediately after treatments was higher than the inactivation rate estimated from the results of the determinations 24 hours post-HPP. In addition, the $logN_{res}$ value, corresponding to the concentration of the resistant tail was lower with the fit of the model to data immediately after HPP. About 5-log reduction were recorded after 5 min of holding time at 600 MPa when measured immediately after HPP. However, a maximum lethality of 3.5 log reduction was recorded when *Salmonella* cells were allowed to recover 24h post-HPP.

The enhancement of the lethality of HPP due to the addition of lactic acid was already seen during the pressure increase phase of the treatment (come-up), resulting in an earlier start of the inactivation curve

from lower initial values compared to the non-acidulated product (although the initial inoculum level of *Salmonella* before HPP was equivalent in both products). The inactivation rate (k_{max}) of *Salmonella* in acidulated products was considerably higher and a greater inactivation of the pathogen was observed before the appearance of the resistance tail. Moreover, the addition of lactic acid contributed to reduce the level at which the resistance tail (residual *Salmonella* concentration) appeared, thus enhancing HPP efficacy.

Interestingly, the differences between the inactivation rate (k_{max}) obtained with *Salmonella* counts immediately after HPP and 24 hours post-HPP were minimized with the addition of lactic acid (Table 2). These results could be associated with the fact that in a more acidic environment, the pressurized *Salmonella* cells could not repair the sublethal damage caused by the HPP treatment during 24 hours in refrigeration (4 °C). From the practical perspective, 5 log reductions could be achieved in the acidulated raw pet food after 3 min at 600 MPa.

4 Conclusions

The study provides scientific data on the HPP-response of *Salmonella*, *E. coli* and *L. monocytogenes*, increasing the knowledge on the variability of the HPP lethal effect. The wide species and strain variability in bacterial HPP inactivation should be considered in risk assessments evaluating the effect of HPP and specifically when validating its efficacy to be used as a control measure within the HACCP plan. The present study has identified some HPP-resistant strains of the pathogens that can be used in challenge tests to assess the efficacy of HPP in raw pet food products. The acidulated formulation enhances the HPP lethality with a variable extent depending on the species and strain. The potential relevance of sublethal injury in the overestimation of the immediate effect of HPP has also been pointed out, which needs to be considered when interpreting the results of validation studies.

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6 Declaration of conflict of interests

Authors declare no conflict of interest. The funders provided the raw materials for preparing the raw pet food product used in the study. They had no responsibility on the design of experiments, data collection and analysis or decision to publish.

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Article 2

High-pressure processing inactivation of *Salmonella* in raw pet food for dog is enhanced by acidulation with lactic acid

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High-pressure processing inactivation of *Salmonella* in raw pet food for dog is enhanced by acidulation with lactic acid

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Abstract

Raw pet food market is growing at rapid rate due to the raising perception as a natural option and the potential health benefits. However, raw pet food also may pose health concerns due to the occurrence of pathogenic bacteria such as *Salmonella* spp. High-pressure processing (HPP) is known as a non-thermal technology to inactivate microorganisms in food, preserving the nutritional characteristics with minimal impact on organoleptic traits. In this framework, the effects of pressure intensity (450-750 MPa), pressure-holding time (0-7 min) and lactic acid concentration (0-7.2 g/kg) on the inactivation of *Salmonella* spp. by HPP in chicken-based raw pet food intended for dogs was evaluated though a central composite design. *Salmonella* reduction ranged from 0.76 to >9 log units depending on the combination of factors, which were all linearly correlated with inactivation. The rate of inactivation slowed down after an initial rapid drop of *Salmonella* levels during treatments, which was reflected as a quadratic term of holding time. The interaction between factors and the quadratic terms of pressure and lactic acid concentration were not statistically significant and therefore not included in the final model. According to the stochastic assessment, after treatments at 500 MPa for 4 min, the probability of a non-acidulated product being contaminated with *Salmonella* decreased to 0.03 %. For these products, an increase in holding-time duration from 4 to 6 min at 500 MPa, decreased the probability of non-conforming products by approximately 50-fold. Remarkably, for products acidulated with 3.6 g/kg of acid lactic, the same increase in treatment duration reduced the probability of non-conforming products in approximately 475-fold. The results highlight the relevant influence of processing parameters and intrinsic factors associated with the product formulation (i.e. lactic acid causing a slight pH decrease) on the lethality of *Salmonella* in pressurized raw pet food. The polynomial model provided constitutes a useful decision-support tool for optimizing HPP of raw pet food, considering matrix acidulation by lactic acid as a strategy to enhance *Salmonella* lethality to comply with current regulations concerning pet food microbiological safety.

Keywords:
HPP, modelling, predictive microbiology, pet food, salmonellosis

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1 Introduction

Raw pet food is composed of pieces of uncooked meat together with animal by-products and vegetables not subjected to thermal treatments, prepared at domestic environments or supplied commercially as fresh, frozen or freeze-dried products (Freeman et al., 2013; Davies et al., 2019). Feeding dogs with products containing raw meat has become a popular practice in recent years, since these products are considered as a more "natural" option in comparison with conventionally processed pet food (Davies et al., 2019; Hellgren et al., 2019). Improvements on pet behaviour, immune function, skin and dental health are among the claimed benefits of raw pet food diets (Joffe & Schlesinger, 2002; Finley et al., 2008).

Regulations of different countries apply zero tolerance regarding the occurrence of *Salmonella* in pet food (European Parliament and Council, 2009; European Commission, 2011; FDA, 2013). Therefore, manufacturers should ensure that raw pet food placed in the market is not contaminated with this pathogen. *Salmonella* prevalence is higher in raw pet food than in conventional processed pet food because raw food does not undergo a lethality process to inactivate bacteria (Hellgren et al., 2019). In Italy, a survey conducted with chicken raw material available for pet food manufacture resulted in the detection of *Salmonella* in 12% of the evaluated samples (Bacci et al., 2019). Van Bree et al. (2018) reported 20% out of 35 commercial samples of raw pet food contaminated with *Salmonella* in the Netherlands. Domesle et al. (2021) reported a turkey-based raw pet food contaminated with three different serovars of *Salmonella.* The occurrence of outbreaks or sporadic cases of animal salmonellosis associated with contaminated dog foods provides evidence of the risk of feeding-*Salmonella* contaminated products to pets (Schotte et al., 2007; Behravesh et al., 2010; Imanishi et al., 2014; Jones et al., 2019).

To limit the health risk for animals due to contaminated raw pet food, high-pressure processing (HPP) is proposed as a non-thermal process to inactivate pathogenic bacteria in this type of products, with minimal impact on nutritional and organoleptic characteristics. It has been demonstrated that the efficacy of HPP to promote bacterial inactivation depends on a series of factors, including processing parameters and matrix related intrinsic factors, e.g., fat, protein, pH and a_w (Hereu et al., 2012; Bover-Cid et al., 2015; Possas et al., 2017; Bover-Cid et al., 2019; Serra-Castelló et al., 2021). However, studies on *Salmonella* inactivation on raw meat-based pet food by HPP are scarce.

Predictive microbiology models are practical tools to understand and quantify the impact of factors that affect microbial behaviour in foods and to optimize the application of technological interventions such as HPP. The survival kinetics of *Salmonella* have been modelled in dry pet food during heat treatment (Rachon et al., 2016) and during long term storage (Lambertini et al., 2016), but to date no modelling approach has been conducted to describe the inactivation of *Salmonella* due to the application of HPP in a raw pet food intended for dog.

In this context, the purpose of the present study was to build and to evaluate a mathematical model describing the inactivation of *Salmonella* in chicken-based raw pet food intended for dogs by HPP as a function of processing parameters, i.e., pressure intensity and holding time, as well as lactic acid concentration as a key parameter of product formulation. The lactic acid was added to lower the pH of raw pet food in order to evaluate to which extent acidulation enhanced pressure-inactivation of *Salmonella*.

2 Material & Methods

2.1 Experimental design

A Central Composite Design (CCD) was performed in order to evaluate the influence of the three variables: pressure intensity (450-750 MPa), pressure-holding time (0-7 min) and lactic acid concentration (0-7.2 g/kg) on the efficacy of HPP treatments to inactivate *Salmonella* spp. in chicken-based raw pet food samples. Twenty-one trials were randomly performed in triplicate in accordance with the CCD, consisting of i) eight trials on factorial points, ii) six trials on axial points, iii) seven trials on the central point to enable the evaluation of the experimental

error and the lack-of-fit of the model. The experimental layout regarding variables and levels is shown in Table 1 and the specific combination of conditions for the twenty-one trials performed are depicted in Table 2.

Table 1. Selected variables (factors) and the corresponding five levels used in the Central Composite Design (CCD).
[a]

Levels[a]	Factors		
	Pressure intensity (MPa)	Holding time (min)	Lactic acid (g/kg)
-1.68	450	0.0	0.0
-1.0	511	1.4	1.5
0	600	3.5	3.6
+1.0	689	5.6	5.7
+1.68	750	7.0	7.2

Considering the circumscribed central composite experimental design for three factors, the scaled value for α relative to the coded values ± 1 was 1.68 ($2^{3/4}$) in order to maintain rotatability and orthogonality.

The ranges set for the technological factors (Table 1), *i.e.* pressure intensities and pressure-holding times, were set based on previous studies, which demonstrated the effectiveness of HPP treatments at 450-750 MPa for up to 7 min to inactivate pathogenic bacteria in foods, including pet food (Jofré et al., 2009; Bover-Cid et al., 2017; Serra-Castelló et al., 2021).

2.2 Bacterial strain and culture preparation

A three-strain cocktail mixture of *Salmonella* Derby CTC1022, *Salmonella* Typhimurium GN0085 and *Salmonella* Enteritidis GN0082, isolated from pork and chicken meat, was used for samples inoculation. These strains were selected based on their higher pressure-resistance in comparison with other 7 *Salmonella enterica* strains tested in a previous screening in which inoculated pet food samples were pressurized at 400 MPa for 5 minutes (Serra-Castelló, et al., 2021). Each strain was grown on Plate Count Agar (PCA, Merck, Darmstadt, Germany) at 37 °C for 18 h. A colony was picked and confluently grown in a new PCA plate at 37 °C for 18 h. Bacterial biomass was collected and resuspended with a cryoprotectant solution consisting of 0.3% of beef extract (Difco Laboratories, Detroit, MI, USA), 0.5% of Tryptone (Oxoid Ltd., Basingtok, Hampshire, UK) and 20% of glycerol and frozen at -80 °C until being used. Cultures were thawed at room temperature before being used. The freeze culture is representative of the status of *Salmonella* in raw materials used to produce the raw pet food, which are usually stored frozen. Moreover, frozen cultures are known to be more resistant to HPP than freshly growth cultures, thus this procedure allow to account for the worse-case scenario (Hereu et al., 2014).

2.3 Raw pet food preparation/formulation

The raw ingredients for pet food manufacture were provided by Affinity Petcare SA and prepared according to a commercial formulation as described in Serra-Castelló et al. (2021). Briefly, raw pet food included chicken (as the main component), vegetables, antioxidants and vitamins and minerals. *Salmonella* was not detected in non-inoculated samples (25 g) of raw pet food. Pet food was prepared in a block format of ca. 10 cm diameter and stored frozen as 1.5 cm-thick slices. Before the experiments, the necessary number of slices were thawed, and lactic acid was incorporated to the samples according to the concentrations set in the CCD (Table 1) by adding the appropriate amount of a lactic acid solution (71 % v/v) kindly provided by CORBION® and kept at 4 ± 1 °C during 24 h before pressurization Samples were inoculated with the *Salmonella* cocktail at a concentration of 10^8-10^9 cfu/g and vacuum-packed in PA/PE bags (oxygen permeability of 50 cm^3/m^2/24 h and a low water vapor permeability of 2.8 g/m^2/24 h; Sistemvac, Estudi Graf S.A., Girona, Spain) 1h before HPP. The a_w and pH of samples were measured before and after HPP treatments with an Aqualab™ equipment (Series 3, Decagon Devices Inc.,

Pullman, WA, USA) and with a penetration 52–32 probe connected to a PH 25 portable pH-meter (Crison Instruments S.A., Alella, Spain), respectively.

2.4 High-pressure processing

Vacuum-packed raw pet food samples were pressurised at the target time-pressure combinations corresponding to the CCD (Table 1). For pressures up to 600 MPa, the equipment used was a Wave 6000 Hiperbaric (Burgos, Spain), while a pilot equipment (Thiot ingenierie, Bretenoux, France – Hiperbaric, Burgos, Spain) was used for pressures above 600 MPa. The come up of pressure was on average 200 MPa/min, while the release was almost immediate. The initial temperature of pressurization fluid (water) was set at 9°C. Compression heating was expected to be about 3 °C/100 MPa (Patazca et al., 2007).

2.5 Microbiological determinations

Raw pet food samples were 10-fold diluted in 0.1 % Bacto Peptone (Difco Laboratories, Detroit, MI, USA) with 0.85 % NaCl (Merck, Darmstadt, Germany) and homogenized for 1 min in a Blender Smasher (bioMérieux, Marcy-l'Étoile, France). The homogenates were serially diluted and plated onto *Salmonella* Plus chromogenic medium (SPCM, CHROMagar™ *Salmonella* Plus; CHROMagar, Paris, France). Colonies were enumerated after incubation at 37 °C for 2 to 5 days (in case of pressurized samples). For expected counts below the detection limit by plate counting (4 cfu/g, resulting from plating 4 ml of homogenate in a 14 cm- diameter plate), the presence of *Salmonella* spp. was investigated in 25 g of sample after selective enrichment of the homogenate in Rappaport-Vassiliadis (RV) broth (Oxoid Ltd., Basingstoke, Hampshire, UK) for 48 h at 41.5 °C. The presence of *Salmonella* in the enriched homogenates was confirmed by PCR using the PrepSEQ™ Rapid Spin Sample Preparation Kit (Applied Biosystems) and MicroSEQ™ *Salmonella* spp. Detection Kit (Applied Biosystems). For modelling purposes, detection of *Salmonella* below the plate detection level was considered -1.0 log cfu/g. Microbiological determinations were conducted in vacuum-packaged samples, pressurized (HPP) or non-pressurized (non-HPP) and either acidulated or non-acidulated in triplicate for each combination of factors considered in the CCD. Vacuum-packaged non-acidulated or acidulated samples that were not pressurized were defined as controls. Inactivation of *Salmonella* spp. in vacuum-packaged pet food samples was expressed in terms of logarithmic reductions as the difference between counts in non-acidulated or acidulated pressurized-samples (N) and controls, i.e., their respective non-acidulated or acidulated non-pressurized samples (N_0), i.e., log (N/N_0).

2.6 Data analysis and statistical modelling

The statistical significance of the differences in the pH of raw pet foods before and after HPP was tested through a t-test. The effects of pressure intensity, pressure holding time and acid lactic concentration on the inactivation of *Salmonella* spp. in raw pet food was investigated by using the Response Surface Methodology. The "rsm" package for R software (R Core Team, 2019) was used to fit quadratic model for each response shown in Equation 1.

$$\log(N/N_0) = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n} \beta_{ii} {x_i}^2 + \sum_{i=j=1}^{n} \beta_{ij} x_i x_j \qquad \text{Equation 1}$$

Where log (N/N_0) is the logarithmic reduction of *Salmonella*; β_0 is a constant; β_i- β_n are model coefficients and x_i-x_n are the independent variables (i.e., pressure intensity, pressure holding time and lactic acid).

To obtain the polynomial equation that best fitted to the experimental data without compromising parsimony, only the significant terms ($p \leq 0.05$) derived from each factor were kept in the final model as indicated by a backward stepwise regression approach. The goodness of fit and the statistical significance of the model were evaluated by means of the root mean square error (RMSE) and the significance of the regression model and the estimated parameters as well as the lack-of-fit test. Response surface graphs were drawn with the value of the independent variable not shown but kept at the central point of the CCD.

2.7 Model performance evaluation

Observed inactivation data (i.e., log reduction) obtained in additional independent experiments were compared with model predictions in order to evaluate its performance. Treatments with foreseeable conditions to be applied at industrial level (EFSA BIOHAZ Panel et al., 2022), i.e., 500 MPa for 4 and 6 min, were applied in products formulated with 3.6 g/kg of lactic acid and products not acidulated. The observed experimental data was compared with model predictions, taking into consideration the 95 % prediction interval of the model. The model was considered acceptable when inactivation observed data were within the 95 % prediction interval of the model.

3 Results

3.1 Reductions of Salmonella spp. in raw pet food due to HPP

The addition of lactic acid in raw pet food at concentrations ranging from 0 to 7.2 g/kg yielded samples with pH varying from 6.97 to 5.72, respectively (Table 2). Differences in *Salmonella* counts between non-acidulated and acidulated samples before HPP were not microbiologically relevant (<0.5 log units). No significant differences were detected between the pH of samples before and after HPP treatments ($p > 0.05$). The a_w of samples was neither affected by HPP application nor the addition of lactic acid and was ≥ 0.99 in all cases.

Table 2. *Salmonella* inactivation on raw pet food samples after high pressure processing treatments at each combination of the Central Composite Design (CCD).

Trial	Pressure (MPa)	Time (min)	Lactic acid (g/kg)[a]	Inactivation (log N/N_0)[b]
1	450	3.5	3.6 (6.08 ± 0.07)	-2.01 ± 0.15
2	511	1.4	1.5 (6.50 ± 0.03)	-0.84 ± 0.07
3	511	1.4	5.7 (5.77 ± 0.02)	-2.21 ± 0.04
4	511	5.6	1.5 (6.50 ± 0.03)	-3.05 ± 0.14
5	511	5.6	5.7 (5.77 ± 0.02)	-4.66 ± 0.08
6	600	0.0	3.6 (6.16 ± 0.04)	-0.76 ± 0.07
7	600	3.5	0.0 (6.97 ± 0.05)	-3.67 ± 0.14
8	600	3.5	3.6 (6.09 ± 0.06)	-5.32 ± 0.25
9	600	3.5	3.6 (6.09 ± 0.07)	-5.59 ± 0.20
10	600	3.5	3.6 (6.22 ± 0.05)	-5.38 ± 0.14
11	600	3.5	3.6 (6.22 ± 0.05)	-5.31 ± 0.20
12	600	3.5	3.6 (6.22 ± 0.05)	-5.49 ± 0.14
13	600	3.5	3.6 (6.22 ± 0.05)	-5.27 ± 0.27
14	600	3.5	3.6 (6.22 ± 0.05)	-5.24 ± 0.51
15	600	3.5	7.2 (5.72 ± 0.08)	-6.80 ± 0.31
16	600	7.0	3.6 (6.08 ± 0.07)	-6.84 ± 0.03
17	689	1.4	1.5 (6.55 ± 0.05)	-4.92 ± 0.29
18	689	1.4	5.7 (5.78 ± 0.10)	-7.42 ± 0.30
19	689	5.6	1.5 (6.55 ± 0.05)	-8.40 ± 1.60
20	689	5.6	5.7 (5.78 ± 0.10)	-8.74 ± 0.88
21	750	3.5	3.6 (6.09 ± 0.05)	-9.33 ± 0.00

[a] Mean ± standard deviation of the pH of samples are reported between parentheses
[b] Mean of three replicates ± standard deviation

Inactivation of *Salmonella* by HPP expressed as log (N/N_0) for each combination of factors of the CCD is shown in Table 2. By increasing both pressure intensity and pressure-holding time, an increase in *Salmonella* inactivation was observed. The maximum reduction achieved in the present experiments was 9.33 log units, when a treatment at the highest pressure level evaluated was applied (i.e. 750 MPa, Trial 21). During this treatment, levels of *Salmonella* decreased to values below plate count detection, although its presence was detected after enrichment of 25 g of the sample. The increase in pressure intensity from 450 to 750 MPa while keeping time and lactic acid concentrations at the central point of the CCD (i.e. 3.5 min and 3.6 g/kg, respectively), increased the inactivation by 7.3 additional log units. Moreover, for treatments at 600 MPa in products containing 3.6 g/kg of lactic acid, an increase in holding time from 0 to 7 minutes resulted in a 6 log reduction (Trials 6 and 16). Considering the addition of lactic acid, an increase from 1.5 to 5.7 g/kg of raw pet food, led to an increase of the HPP inactivation by 1.4 additional log units of reduction in treatments at 511 MPa/1.4 min (Trials 2 and 3). The same increase in lactic acid concentration at 689 MPa/1.4 min resulted in an acid-related reduction of *Salmonella* of 2.5 log (Trials 17 and 18). In these experiments, the increase in lactic acid concentrations reduced the pH of raw pet food samples from 6.5 to 5.8 (Table 2).

3.2 Modelling the inactivation of Salmonella spp. in raw pet food by HPP

The coefficients of the empirical model (Equation 1) quantifying the relationship between the *Salmonella* inactivation in raw pet food and the independent factors evaluated, i.e. pressure, pressure-holding time and lactic acid concentration, are shown in Table 3. The model is statistically significant as indicated by the *F*-value = 268.1 ($p \leq 0.00001$) and the non-significant lack-of-fit test (*F*-value = 5.2; $p > 0.05$). Moreover, the low RMSE value of 0.677 indicated a satisfactory goodness of fit.

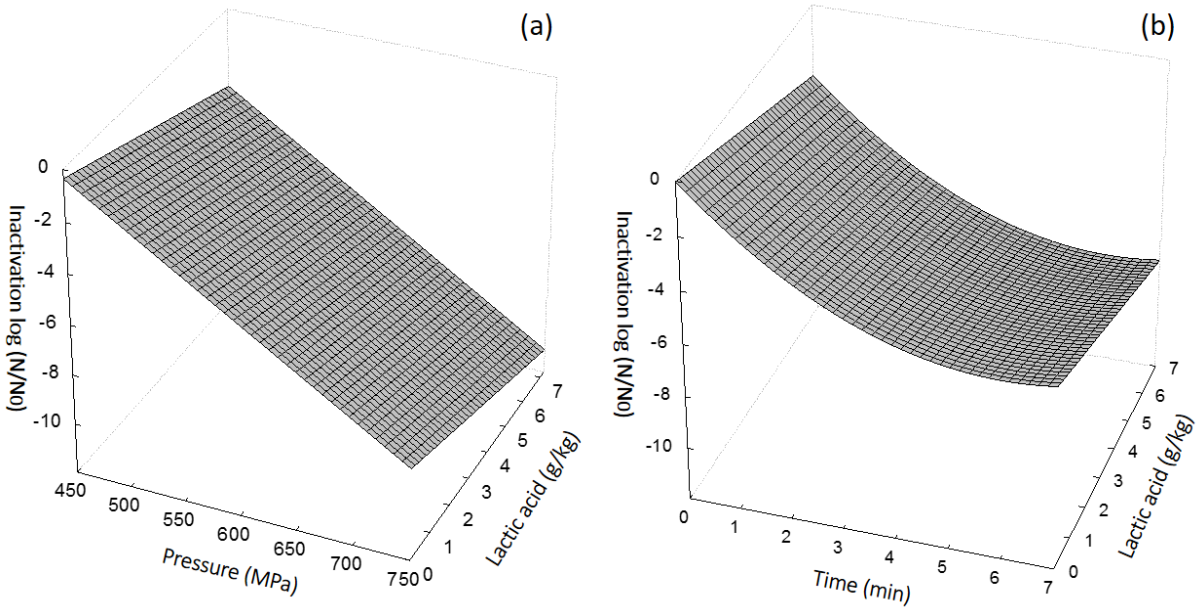

Figure 1. Response surface graphs of high-pressure processing (HPP)-induced inactivation of *Salmonella* spp. in raw pet food according to the developed model. (a) Pressure intensity and lactic acid concentration effects; (b) Holding time and lactic acid concentration effects. The factors not included in each graph are maintained at the central value of the central composite design; time = 3.5 min in graph (a) and pressure = 600 MPa in graph (b).

The response surface graphs generated based in the obtained model are shown in Figure 1. The three factors evaluated were positively correlated with the inactivation of *Salmonella* spp. in raw pet food and are present in the model as linear terms ($p \leq 0.05$). Effect estimates indicated that pressure intensity was the quantitatively most important factor influencing inactivation, followed by pressure-holding time. Interactions between the factors were not significant ($p > 0.05$) and thus not included in the final model.

A non-linear relationship between *Salmonella* inactivation and pressure-holding time was marked and reflected by the presence of a quadratic term in the model. It means that by increasing the duration of pressure treatments, there is a slowing down on reductions, with higher inactivation rates at the beginning of pressurization (Figure 1a and 1b). The results of model performance evaluation are shown in Table 4. The model could be successfully applied to predict the inactivation of *Salmonella* in raw pet food containing 0 or 3.6 g/kg of lactic acid treated at 500 MPa for 4 and 6 min, as independent data obtained in additional experiments carried out at these conditions fall within the 95 % prediction interval of the model.

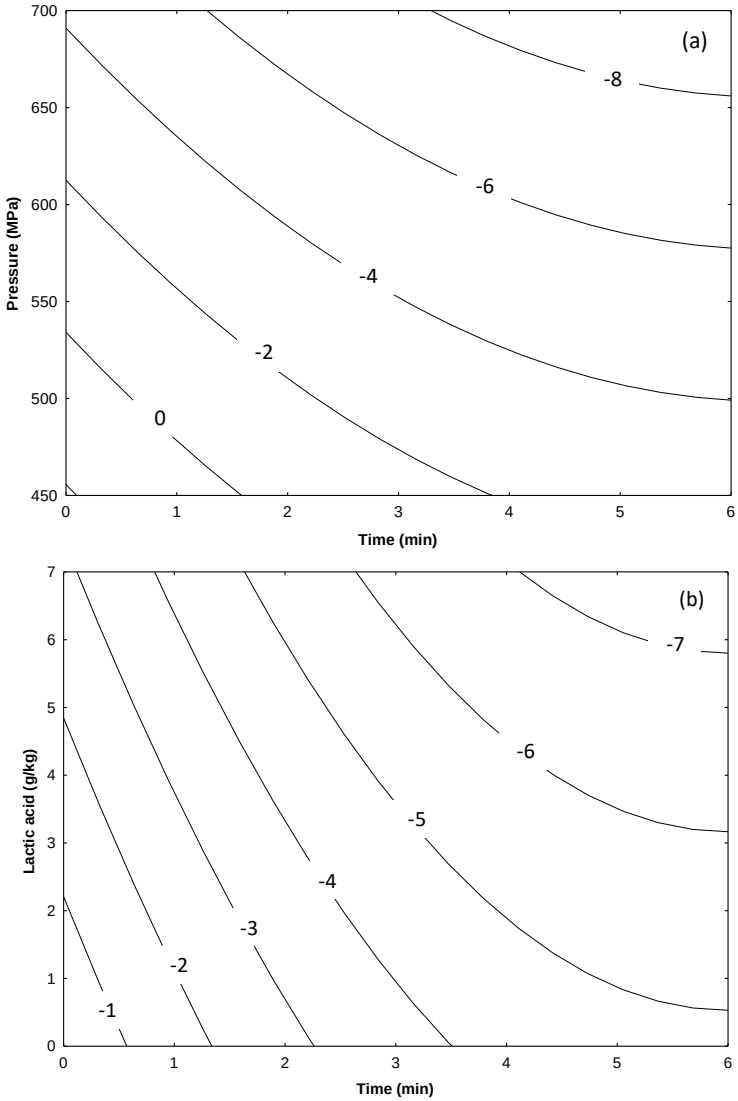

Figure 2. Contour plots describing the inactivation effect of high-pressure processing (HPP) in raw pet food at different combinations of (a) pressure intensity and pressure-holding time at a lactic acid concentration = 4 g/kg of raw pet food and (b) lactic acid and pressure-holding time at 600 MPa. Numbers in each line indicate the inactivation value, i.e. log (N/N_0).

The contour plot showing the combination of pressure intensity and holding time that allow to accomplish a target isoreduction level in raw pet food containing 4 g/kg of lactic acid is shown in Figure 2a. It can be deduced by checking the plot that by applying treatments at 500 MPa for 6 min, a 4 log reduction in *Salmonella* levels would be achieved. Additionally, to achieve a 6 log reduction at 600 MPa, treatment duration might be at least of 4 min.

4 Discussion

4.1 Salmonella spp. inactivation in raw pet food by HPP

The results of the present study highlighted the role of processing parameters on the lethality of HPP, as reported in previous investigations in foods other than raw pet food (Bover-Cid et al., 2017; Possas et al., 2017). Moreover, they revealed that the HPP-resistance of *Salmonella* in chicken-based raw pet food was lower in comparison with dry-cured meat products and comparable to the inactivation levels achieved with the pressurization of the pathogen in liquid matrices or culture broth. *Salmonella* reductions in the range of 4-8 log were reported after pressurization of culture broth at 350-550 MPa up to 10 min (Lee & Kaletunç, 2010; Maitland et al., 2011), while notably lower reductions, within the range 2-4 log, were reported in dry-cured ham (with a a_w of 0.88) subjected to 450-750 MPa for 5 min. These differences would be associated with the protective effect of the low a_w of the matrix on the lethality of HPP on *Salmonella*, since higher microbial reductions have been quantified in matrices with higher a_w, such as the raw pet food under study (a_w > 0.99) (Bover-Cid et al., 2015; Georget et al., 2015). Besides the effect of a_w, additional reductions in raw pet food in comparison with other meat products can be associated with the pH decrease through the addition of lactic acid which could be explained by the lower resistance of pathogens to HPP in more acidulated conditions (Alpas et al., 2000).

Due to the lack of studies dealing with the pressure-induced inactivation of *Salmonella* in raw pet food, comparison of results with data obtained during raw poultry pressurization seems reasonable, since chicken meat is the main ingredient of the raw pet food under study (80 % w/w). Reductions of 3.35 and 3.5 log in *Salmonella* levels were achieved after the pressurization at 450 MPa for 5 min of inoculated ground chicken (Sheen et al., 2015) and chicken fillets (Kruk et al., 2011), respectively. In line with these investigations, in the present study the application of 450 MPa for a slightly shorter time yielded a slightly lower log reduction (2 log, Trial 1).

In the present study, acidulation by adding acid lactic was effective in increasing *Salmonella* inactivation. Besides acidulation, additional control measures can be applied together with HPP to promote the inactivation of *Salmonella* and to avoid the growth of pressure-injured cells during storage of raw pet food, including refrigeration of pressurized products (Jofré et al., 2010; Lerasle et al., 2014). For instance, Morales et al. (2009) found no recovery of pressure-injured cells of *Salmonella* in chicken fillets subjected to treatments at 300 and 400 MPa for up to 20 min during the subsequent storage at 4 °C for 72 hours. Therefore, the storage of pressurized raw pet food under refrigeration according to manufacture recommendations would assist the compliance with current regulations for *Salmonella*.

The non-linear relationship between *Salmonella* and pressure-holding time found in the present Article is compatible with the occurrence of a tail of resistant cells which may indicate the presence of subpopulations of *Salmonella* with different susceptibilities to pressure (Tamber, 2018). The same non-linear trend was observed in other studies modelling the microbial pressure-induced inactivation in foods (Hereu et al., 2012; Tananuwong et al., 2012; Lerasle et al., 2014). From the technological point of view, the occurrence of a tail during microbial inactivation has remarkable implications. Since the inactivation rate in the tail part is drastically reduced, no significant additional *Salmonella* reductions would be achieved by increasing processing times, which means that additional operational costs derived from increased pressure-holding times could be avoided. Based on capital costs, an economically reasonable holding time to be applied at industrial level was estimated in a maximum of 6 min (Garriga et al., 2004).

Table 3. Results of the multivariate regression analysis describing the effect of pressure intensity, pressure-holding time and lactic acid concentration on the inactivation of *Salmonella* spp. in raw pet food.

Terms[a]	Regression coefficients	Standard Error	*t*-value	*p*-value	RMSE[b]
Intercept	15.1380	0.6545	23.1293	<0.0001	0.677
P (MPa)	-0.0255	0.0010	-25.9814	<0.0001	
t (min)	-1.5467	0.1412	-10.9741	<0.0001	
LA (g/kg)	-0.3795	0.0410	-9.2495	<0.0001	
t^2 (min)	0.1219	0.0191	6.3613	<0.0001	

[a]: *P*, pressure; *t*, holding time; *LA*, lactic acid concentration

[b]: root mean square error (RMSE)

On the other hand, regarding food safety, the occurrence of a tail of resistant cells is a concern during the subsequent storage and handling practices. Even if resistant cells may be sublethally damaged, they can recover and initiate growth if the intrinsic and storage conditions are favourable (Hereu et al., 2014).
Recommendations regarding the required lethality of HPP treatments to eliminate *Salmonella* in raw pet food have not been established. However, the application of technologies alternative to the thermal treatment such as HPP must ensure the reduction of the loads of pathogenic microorganisms in foods in about 4 to 6 log reductions (IFT, 2002). Considering that a HPP treatment should assure those reductions of *Salmonella* in raw pet food, the model developed in this study can be applied, for instance, to set the appropriate processing parameters, assuming the addition of a fixed lactic acid concentration.
On the other hand, according to the requirements established in the US for the production of fully cooked poultry products, a lethality process which must include a cooking step may assure a 7-log reduction of *Salmonella* (CFR, 2018). Simulations using the developed model indicate that this target inactivation would only be achieved in raw pet food formulated with lactic acid. For example, a 7-log reduction would be achieved when applying a treatment at 600 MPa for at least 4.2 min in raw pet food containing 7 g/kg of lactic acid (Figure 2b). By reducing the lactic acid concentration to 6 g/kg, the minimum holding time of a HPP treatment at 600 MPa required to achieve the target inactivation would increase to 5 min (Figure 2b). Therefore, the model developed in the present study can be applied to define HPP parameters and lactic acid concentrations required to achieve desired levels of *Salmonella* inactivation, being an important tool for process assessment and optimization in view of food safety assurance.

4.2 Validation of HPP as a killing step in raw pet food using the FSO concept

The validation of a control measure provides evidence that a specific process will result in products that meet microbiological and quality requirements (Zwietering et al., 2010). Considering that there is no specification of the number of *Salmonella* reductions that may be reached during HPP treatments applied to pet food, the management of the food safety of this product can be approached through Food Safety Objective (FSO) concept (ICMSF, 2002). In the present study a stochastic approach (Zwietering et al., 2010) was used to evaluate the probability that HPP treatments would result in products that comply with current regulations concerning *Salmonella* in pet food. The FSO is the maximum level of the pathogen that are tolerated at the moment of consumption and can be calculated by means of Equation 2.

$$H_0 - \sum R + \sum I \leq FSO \qquad \text{Equation 2}$$

where H_0 is the initial level of *Salmonella* contamination in raw pet food; ΣR is the total reduction of *Salmonella* during processing, e.g. by HPP application; and ΣI is the total *Salmonella* increase (growth and/or recontamination) during the whole process.

To determine whether a food batch meets an FSO, the distribution of initial levels of the pathogen (H_0) within a food must be understood (van Schothorst et al., 2009). The initial *Salmonella* concentration in chicken-based raw pet food was estimated by applying the probabilistic approach published by Valero et al. (2014) based on presence/absence data provided by the pet food producer and was described by a normal distribution with mean -1.55 log cfu/g and standard deviation 0.51 log cfu/g.

Table 4. Results of additional HPP experiments conducted for the evaluation of the model performance to describe de pressure-induced inactivation of *Salmonella* in raw pet food.

Pressure (MPa)	Time (min)	Lactic acid (g/kg)	Observed inactivation (log N/N_0)	Predicted inactivation (log N/N_0)	-95 % PI (log N/N_0)	+95 % PI (log N/N_0)
500	4	0	-1.64 ± 0.12	-1.86	-3.05	-0.67
500	6	0	-2.09 ± 0.06	-2.51	-3.73	-1.31
500	4	3.6	-2.25 ± 0.24	-3.22	-4.37	-2.07
500	6	3.6	-2.83 ± 0.19	-3.88	-5.06	-2.71

PI = Prediction interval

Growth of *Salmonella* and recontamination after HPP treatments were deemed negligible (i.e., ΣI = 0) since products were pressurized in their package and after HPP they stored frozen or under refrigeration temperatures not supporting the growth of *Salmonella* (ICFMH, 1996). *Salmonella* reduction observed in HPP treatments were expressed as normal distributions (ΣR, Table 5). The *FSO* was set at < -1.41 log cfu/g, which corresponds to the logarithm of 1 cfu in 25 g of product, the maximum level of *Salmonella* in accordance with regulations that require no detection in 25 g of product. It is assumed that 95% of the distribution of concentration must satisfy the test limit so that the FSO is met.

The stochastic assessment indicated that a high number of contaminated product units could be present in a lot, i.e., up to ca. 38 %. The percentage of non-conforming products regarding the *FSO* and the overall distribution of *Salmonella* in acidulated and non-acidulated products subjected to pressurization are shown in Table 5. After treatments at 500 MPa/4 min, the probability of a non-acidulated product being contaminated with *Salmonella* decreased to 0.03 %. For these products, an increase in holding-time duration from 4 to 6 min at 500 MPa, decreased the probability of non-conforming products in approximately 50-fold (Table 5). Remarkably, for products acidulated with 3.6 g/kg of acid lactic, the same increase in treatment duration reduced the probability of non-conforming products in approximately 475-fold.

Table 5. Stochastic evaluation of zero tolerance compliance regarding Salmonella spp. (i.e. no detection in 25g) in high pressure processed raw pet food.

Pressure (MPa)	Time (min)	Lactic acid (g/kg)	Initial contamination (H_0, log cfu/g)	Observed inactivation (ΣR, log N/N_0)	H_0-ΣR+ΣI (log cfu/g)	P (x > FSO) %
500	4	0	-1.55 ± 0.51	-1.64 ± 0.12	-3.19 ± 0.52	0.0313
500	6	0	-1.55 ± 0.51	-2.09 ± 0.06	-3.64 ± 0.51	0.0006
500	4	3.6	-1.55 ± 0.51	-2.25 ± 0.24	-3.80 ± 0.56	0.0010
500	6	3.6	-1.55 ± 0.51	-2.83 ± 0.19	-4.38 ± 0.54	0.0000

By increasing the acid lactic concentration from 0 to 3.6 g/kg and applying 500 MPa for 4 min, the probability of non-conforming units was reduced by approximately 30-fold, while the same increase in lactic acid concentration in parallel with the increase in pressure-holding time from 4 to 6 minutes would reduce the prevalence of *Salmonella* expressed as percentage of contaminated units per batch to approximately 0.

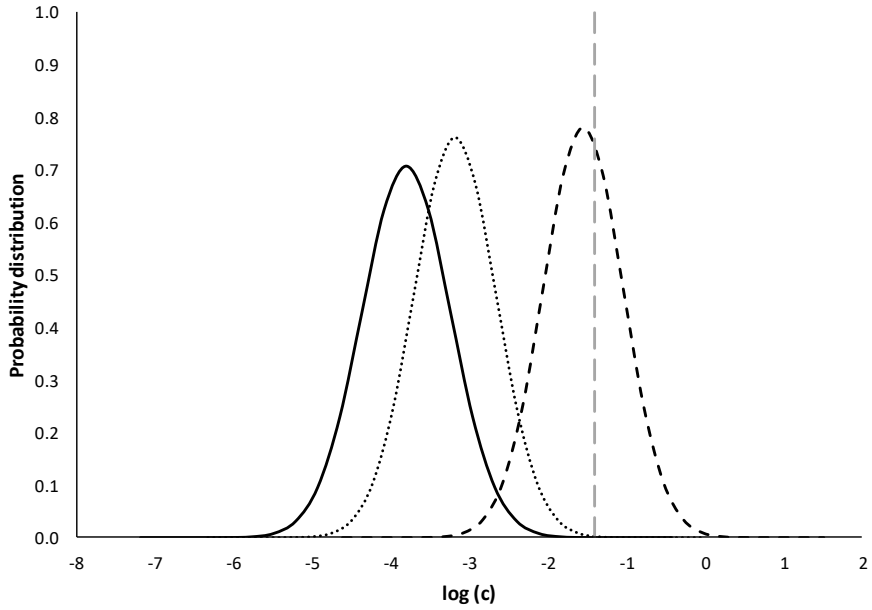

Figure 3. Probability distribution of the initial level of contamination of *Salmonella* (log cfu/g) in chicken-based raw pet food (H_0, – – –) and after pressurization at 500 MPa for 4 min of products acidulated with 3.6 g/kg (——) and non-acidulated products (·····). The vertical dashed line indicates the FSO < -1.4 log cfu/g.

The impact of acidulation and HPP treatments in the distribution of *Salmonella* in raw pet food can be seen in Figure 3, where it can be noted that the distribution of *Salmonella* in acidulated products is shifted to the left of the graph, representing lower concentrations.

5 Conclusions

The inactivation of *Salmonella* spp. by HPP in chicken-based raw pet food intended for dogs was dependent of the pressure intensity and holding time and could be notably enhanced by the lactic acid addition in the product formulation. By increasing the values of the three factors, higher inactivation is quantified, although the inactivation rate significantly decreases at holding times of 4-6 min due to the occurrence of a tail of pressure-resistant cells, which should be considered not only from the food safety point of view but from the operational and economic perspective. The model developed in the present study is suitable to assess and optimize the impact of HPP conditions. The model constitutes a useful decision support tool to assist pet food producers on setting appropriate combinations of processing parameters and lactic acid concentrations on raw chicken-based pet food formulations to achieve desired levels of *Salmonella* inactivation to assure the compliance with the microbiological criteria regulation.

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7 Declaration of conflict of interests

Authors declare no conflict of interest. The funders provided the raw materials for preparing the raw pet food product used in the study. They had no responsibility on the design of experiments, data collection and analysis or decision to publish.

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Article 3

High pressure processing to control *Salmonella* in raw pet food without compromising the freshness appearance: the impact of acidulation and frozen storage

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High pressure processing to control *Salmonella* in raw pet food without compromising the freshness appearance: the impact of acidulation and frozen storage

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Abstract

The trend of feeding dogs and cats with raw pet food claiming health benefits, poses health concerns due to the occurrence of pathogenic bacteria. High pressure processing (HPP) allows the non-thermal inactivation of microorganisms, preserving the nutritional characteristics with minimal impact on organoleptic traits of food. The present study aimed to evaluate and model the effect of HPP application (450-750 MPa for 0-7 min) on the inactivation of *Salmonella*, endogenous microbiota and colour of raw pet food formulated with different concentrations of lactic acid (0-7.2 g/kg) as natural antimicrobial. Additionally, the effect of a subsequent frozen storage of pressurised product was assessed.
Salmonella inactivation ranged between 1 and 9 log, depending on the combination of conditions. According to the polynomial model obtained, the effect of pressure was linear, while a quadratic term was also included for holding time (depicting the occurrence of a resistant tail at *ca*. 4 to 6 min). The effect of lactic acid was dependent on the pressure level, being most relevant for treatments below 600 MPa. Frozen storage after HPP prevented the pathogen recovery and caused a further *Salmonella* inactivation enhanced by lactic acid in most of the treatments. Endogenous microbial groups were significantly reduced by HPP to below the detection level in several conditions. In general, little effect of HPP on the instrumental colour parameters was observed, except for a slight increase in lightness, which was hardly appreciable from visual observation.
High pressure processing emerges as a relevant technology for the control *Salmonella* spp. and manage the microbiological safety of raw pet food. The mathematical model can be used as decision support tool to design safer of raw pet food, while keeping the desired freshness appearance of the products.

Keywords:
raw pet food, high hydrostatic pressure, mathematical modelling, predictive microbiology, salmonellosis.

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1 Introduction

Raw meat-based diets (RMBD) for pets are mainly composed by uncooked animal products or by-products, vegetables, fruits and/or grains (Nüesch-Inderbinen et al., 2019). They can be home-prepared or commercially supplied on their fresh, frozen or freeze-dried form or as premixes intended to be complemented with raw meat (van Bree et al., 2018; Davis et al., 2019; Nüesch-Inderbinen et al., 2019). Feeding cats and dogs with RMBD has become a popular practice by pet owners, due to their more "natural" and fresh characteristics and the perceived healthier benefits, including improvement of skin and coat and increase in oral health of pets, compared with cooked (sterilised) or dry pet food options (Weese et al., 2005; Fredriksson-Ahomaa et al., 2017; Davis et al., 2019). Despite the claimed benefits of feeding pets with RMBD, this practice may pose health risks to animals, as raw materials may be contaminated with enteric pathogens such us *Salmonella* (Fredriksson-Ahomaa et al., 2017; Giacometti et al., 2017). In surveys conducted to evaluate the presence of bacterial pathogens in Dutch and Canadian commercially available RMBD, *Salmonella* was present in 20 % of raw pet food samples (Weese et al., 2005; van Bree et al., 2018). Whole genome sequencing approach found clinical isolates of *Salmonella* obtained from sick cats and dogs to be closely related to *Salmonella* strains isolated from raw pet food (Jones et al., 2019). In this context, current regulations require that commercial suppliers must ensure *Salmonella* is not detected in raw pet food (European Commission, 2011; FDA, 2013).

The supplementation of pet food with acid lactic has demonstrated to promote oral health in cats, inhibiting dental plaque, calculus and tooth stain accumulation (Scherl et al., 2019). Besides the health benefits, it has been demonstrated that the acidulation with lactic acid can be effective to control pathogenic bacteria such as *Salmonella*, *Escherichia coli* and *Listeria monocytogenes* in raw pet food samples (Serra-Castelló et al., 2022).

High Pressure Processing (HPP) technology is an emerging strategy being implemented by pet food producers as a killing step to assure compliance with current microbiological regulations (Anonymous, 2019). The application of high levels of pressure during few minutes can inactivate microorganisms in foods, with a minimal impact on their organoleptic and nutritional characteristics (Bover-Cid et al., 2017; Possas et al., 2017). In addition, frozen storage after HPP application has shown to enhance the inactivation of pathogens in some types of foods, including strawberry puree (Huang et al., 2013) and ground beef (Black et al., 2010).

The combination of preservation technologies such as HPP with other preservation factors like acidulation and frozen storage to produce safe, stable and high quality food products has been designated as the "hurdle concept" (Leistner and Gorris, 1995). To date, the impact of combining these hurdles on the microbiological quality of RMBD has not been evaluated.

This work aimed at evaluating the inactivation of *Salmonella* and endogenous microbiota in raw pet food intended for cats, treated by HPP associated with acidulation with acid lactic and its subsequent frozen storage. The effects of these hurdles on raw pet food instrumental colour were also evaluated.

2 Material & Methods

2.1 Experimental design

A Central Composite Design (CCD) was performed to evaluate the impact of pressure level (450-750 MPa), holding times (0-7 min) and lactic acid concentrations (0-7.2 g/kg), on the efficacy of HPP treatments to inactivate *Salmonella* spp. and endogenous microbiota in raw pet food samples. The experimental layout performed is depicted in Table 1. The ranges set for the pressurization parameters, *i.e.* pressure levels and holding times, were set based on previous studies that demonstrated the effectiveness of HPP treatments at pressure levels of 450-750 MPa and holding times of 0 (i.e. a pulse of pressure come-up followed by immediate release) up to 7 min to inactivate pathogenic bacteria in foods (Bover-Cid et al., 2015; 2017). 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 trials were randomly performed to minimize the systematic bias due to disturbing effects of environmental conditions (Robinson, 2000; Barba et al., 2014).

2.2 Raw pet food preparation/formulation

The raw ingredients for pet food intended for cat were provided by Affinity Petcare SA. and included: chicken, plant based-ingredients, salmon and spices. Pet food was prepared at the pilot plant according to a commercial recipe and procedure and stored frozen at -20 °C until being used. The proximal composition of the raw pet food was: moisture (70 %), protein (12 %), fat (6 %), ash (2 %) and fibre (1%).
Lactic acid was added to samples at the concentrations set in the CCD (Table 1) by adding the appropriate amount of a 71 % lactic acid solution kindly provided by CORBION®. This procedure was conducted 24 hours before pressurization in order to allow the stabilization of the pH.

2.3 Salmonella strains, culture preparation and inoculation

Samples were inoculated with a three-strain *Salmonella* cocktail composed of equal amounts of *Salmonella* Derby CTC1022, isolated from pork meat, and *Salmonella* Typhimurium GN0085 and *Salmonella* Enteritidis GN0082, isolated from chicken meat. Strain selection was based on previous HPP-resistance studies (Serra-Castelló et al., 2022). For the preparation of the cocktail, individual cultures of the selected strains were prepared as reported in Serra-Castelló et al. 2022. Briefly, a loopful of the frozen stock culture (-80 °C) was streaked on Plate Count Agar (PCA, Merck, Darmstadt, Germany) at 37 °C overnight (18 h). An individual pure colony was spread in a new plate of PCA and grown at 37 °C overnight to reach the stationary growth phase, which makes *Salmonella* more resistant than in the exponential growth phase. Bacterial biomass on the surface of the PCA plate was collected, resuspended with a cryoprotectant solution (0.3% of beef extract (Difco Laboratories, Detroit, MI, USA), 0.5% of Tryptone (Oxoid Ltd., Basingtok, Hampshire, UK) and 20% of glycerol) and frozen at -80 °C until being used. The frozen culture is representative of the status of the strain in raw materials usually stored frozen to produce the raw pet food and, in addition, it is known to protect pathogens from HPP, making this procedure a conservative approach to cover worst-case scenarios (Hereu et al., 2014).
Samples were inoculated with the *Salmonella* cocktail (1% v/w) just before pressurization.

2.4 High pressure processing and storage conditions

Twenty-five-gram samples of the inoculated raw pet food were vacuum-packed in PA/PE plastic bags (oxygen permeability of 50 cm^3/m^2/24 h and a low water vapor permeability of 2.8 g/m^2/24 h; Sistemvac, Estudi Graf S.A., Girona, Spain) and pressurised at the target time-pressure combinations established by the CCD (Table 1). For pressures up to 600 MPa, the equipment used was a Wave 6000 from Hiperbaric S.A. (Burgos, Spain), while a pilot equipment from Thiot ingenierie – Hiperbaric (Bretenoux, France – Burgos, Spain) was used for pressure levels above 600 MPa. The average pressure come-up time was 191 MPa/min, while the pressure release was almost immediate (< 5s). The initial temperature of the pressurization fluid was set at 9 °C. Compression heating was expected to be about 3 °C/100 MPa (Patazca et al., 2007), therefore no thermal effect was expected. HPP samples were stored frozen (-18 °C) for 14 days.

2.5 Sampling and microbiological determinations

Microbiological determinations of samples inoculated with *Salmonella* and non-inoculated samples were conducted in triplicate for each trial of the CCD before HPP, immediately after the HPP and after 14 days of frozen storage. Frozen-stored samples were thawed at 4 °C for 24 hours before microbiological analysis in order to reproduce the recommendations of the raw pet food manufacturer regarding storage and thawing at household environments prior to consumption.
Raw pet food samples inoculated with *Salmonella* were ten-fold diluted in 0.1 % Bacto Peptone (Difco Laboratories, Detroit, MI, USA) with 0.85 % NaCl (Merck, Darmstadt, Germany) and homogenized for 60 seconds in a Smasher™ bag blender (bioMérieux, Marcy-l'Étoile, France). The homogenates of inoculated samples were serially diluted and plated onto *Salmonella* Plus chromogenic medium (SPCM, CHROMagar™ *Salmonella* Plus;

Scharlab, S.L., Sentmenat, Spain). Colonies were enumerated after incubation at 37 °C for at least 48h (i.e. number of colonies were checked daily up to 5 days) to allow the recovery of cells sublethally injured due to the HPP treatments. For expected counts below the enumeration limit (< 2.5 cfu/g; no colony after spreading 4 ml of 1:10 dilution), the presence or absence of *Salmonella* spp. was determined after an enrichment of the homogenate in Rappaport-Vassiliadis (RV) broth (Oxoid Ltd., Basingstoke, Hampshire, UK) for 48 h at 42 °C. The enriched homogenate was streaked onto SPCM plates. The presence of *Salmonella* in the enriched homogenates was confirmed by PCR using the PrepSEQ™ Rapid Spin Sample Preparation Kit (Applied Biosystems) and MicroSEQ™ *Salmonella* spp. Detection Kit (Applied Biosystems).

Non-inoculated raw pet food samples were used to determine the levels of endogenous microbiota i.e. total aerobic mesophilic bacteria, *Enterobacteriaceae*, *Pseudomonas* spp. and lactic acid bacteria (LAB) before and after HPP. Raw pet food samples were ten-fold diluted in 0.1 % Bacto Peptone (Difco Laboratories, Detroit, MI, USA) with 0.85 % NaCl (Merck, Darmstadt, Germany) and homogenized for 60 seconds in a Smasher^{TM} blender (bioMérieux, Marcy-l'Étoile, France). *Enterobacteriaceae* were enumerated on VRBD (Violet Red Bile Dextrose) agar (Merck Life Science S.L.U, Madrid, Spain) incubated for 24 hours at 37 °C. *Pseudomonas* spp. were enumerated on Pseudomonas CFC selective agar (Oxoid S.A., Madrid, Spain) incubated at 25 °C for 48 hours. Total aerobic mesophilic bacteria was plated on PCA (Plate Count Agar; Merck Life Science S.L.U, Madrid, Spain) and incubated at 30 °C for 72 hours. LAB was plated MRS (de Man, Rogosa and Sharpe) agar (Merck Life Science S.L.U, Madrid, Spain) and incubated at 30 °C for 72 hours under anaerobiosis (AnaeroGen 2.5l, Thermo Scientific-Oxoid).

2.6 Physico-chemical and instrumental colour measurements

The a_w of the samples was measured with an Aqualab™ equipment (Series 3, Decagon Devices Inc., Pullman, WA, USA) and the pH was measured with a PH25 pHmeter (Crison Instruments S.A., Alella, Spain) before and after HPP treatments.

Instrumental colour was assessed as the most determinant and sensitive measurement of potential changes of the product appearance due to HPP. Instrumental colour measurement consisted of L^* (lightness), a^* (redness) and b^* (yellowness) before (L_0^*, a_0^* and b_0^*) and after (L^*, a^* and b^*) the HPP treatment using a colorimeter (Minolta Chroma Meter CR-400, Tokyo, Japan) with illuminant D65 with 2 °C viewing angle and calibrated using a standard white tile. Measurements were conducted in triplicate for each condition of the CCD. To provide the relevance of the difference seen between the colour before and after HPP, the total colour change (Δ E) was calculated according to Eq-1.

$$\Delta E = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2} \quad \text{(Eq. 1)}$$

2.7 Data analysis & modelling

Inactivation of *Salmonella* spp. and endogenous microbiota in pet food samples was expressed in terms of logarithmic reductions as the difference between counts after HPP treatments (N) and before treatments (N_0), *i.e.*, log (N/N_0). For modelling purposes, *Salmonella* positive results below the detection limit were recorded as -1.40 log cfu/g. For the colour the change of the colour parameters (ΔL^*, Δa^*, Δb^*) was quantified as the difference between measurements after (L^*, a^* and b^*) and before (L_0^*, a_0^* and b_0^*) HPP treatments, i.e., $L^* - L_0^*$, $a^* - a_0^*$, $b^* - b_0^*$.

The effect of pressure level, holding time, lactic acid concentration and their possible interactions on the inactivation of *Salmonella* spp., endogenous microbiota and colour in raw pet food was investigated by using the Response Surface Methodology (RSM). The "rsm" package for R software (R Core Team, 2019) was used for stepwise backward regression.

To obtain the polynomial equation that best fitted the experimental data without compromising parsimony, only the significant terms derived from each factor were kept in the final model as indicated by an ANOVA test ($p \leq 0.05$). The ANOVA was performed to estimate the coefficients of the final equation. The goodness-of-fit was

evaluated by means of the root mean square error (RMSE) that measures the differences between the fitted and observed inactivation values. The statistical significance of the model was evaluated through the significance of the *p*–values derived from the *F*–test. Response surface graphs were drawn in which the value of the not shown independent variable was kept at the central point of the CCD.

3 Results and Discussion

3.1 Salmonella inactivation due to HPP and lactic acid

HPP inactivation of *Salmonella*, expressed as log reductions, for each combination of the CCD immediately after the HPP treatment is shown in Table 1.

The highest *Salmonella* inactivation (9.08 log reduction) was observed in trial 23 where the highest pressure (750 MPa) was applied. On the contrary, in trials 2 (shortest HPP treatment) and 6 (samples without lactic acid) the lowest *Salmonella* inactivation were recorded (1.11 and 1.10 log, respectively), indicating that the three parameters studied in the present work (pressure level, holding time and lactic acid concentration) were relevant to explain the inactivation of *Salmonella* in raw pet food due to the HPP. However, lactic acid alone was not capable to reduce *Salmonella* counts as the level of contamination before HPP was very similar in all trials in agreement with the target inoculation level. For instance, counts of *Salmonella* before HPP were 8.26 ± 0.07 Log_{10} cfu/g in samples without lactic acid (trial 7) and 8.23 ± 0.04 Log_{10} cfu/g in the samples with the highest tested lactic acid amount (7.2 g/kg in trial 17).

The pressure-resistance of *Salmonella* in raw pet food observed in the present study was higher than that reported in simpler matrixes such as laboratory media (Alpas et al., 2000) and fresh raw chicken (Tananuwong et al., 2012), which is in line with the recognised protective effect of complex food matrixes on the microbial inactivation during HPP (EFSA BIOHAZ et al., 2022).

According to the modelling results, the pressure effect on *Salmonella* inactivation was almost linear, indicating that the inactivation of the pathogen was directly proportional to the level of pressure applied (Table 2). In this line, Cap et al. (2020) also reported higher inactivation of *Salmonella* with increasing pressure levels (100-600 MPa) in frozen chicken breast. The holding time parameter contributed to the *Salmonella* inactivation model with a linear and a quadratic term. These results indicated that after a rapid linear-based decrease on *Salmonella* levels during the first minutes of treatment, approximately 4-6 min, there was a slowing down on inactivation due to a strong reduction of the pressure- inactivation rate. This phenomenon, is compatible with the occurrence of a tailing of resistant cells, as already observed in the inactivation kinetics of the same *Salmonella* strains during pressurization at 600 MPa in chicken based raw pet food (Serra-Castelló et al., 2022). *Salmonella* resistance tails were empirically observed in a wide range of pressure levels and lactic acid concentrations.

The addition of lactic acid in raw pet food at concentrations ranging from 0 to 7.2 g/kg yielded samples with pH varying from 6.80 to 5.55, respectively (Table 1). Significant differences between the pH of the samples before and after HPP treatments were not detected ($p > 0.05$). The a_w of samples was neither affected by HPP application and was ≥ 0.99 in all cases. The increase in lactic acid concentrations enhanced the lethal effect of HPP treatment. The membrane damage in bacterial cells induced by HPP could enable the entry of antimicrobial substances that can enhance lethality of pressure treatments (García-Graells et al., 1999). Moreover, Jung et al. (2013) reported that increasing levels of lactic acid (pH 4.0-6.0) enhanced the inactivation of *L. monocytogenes* after pressurization at 300 MPa for 5 min.

Table 1. *Salmonella,* aerobic mesophilic and lactic acid bacteria inactivation (log reduction) and changes in the colour lightness (*L**), redness (*a**) and yellowness (*b**) on raw pet food samples due to high pressure processing (HPP) treatments at each combination of the Central Composite Design.

Trial	Lactic acid (g/kg)[a]	Pressure (MPa)	Time (min)	Microbial inactivation (log reduction)[b]				Colour changes post HPP[c]			
				Salmonella		Mesophilic bacteria	Lactic acid bacteria				
				Post-HPP	Post-HPP + Frozen storage	Post-HPP	Post-HPP	$L^*- L_0^*$	$a^*- a_0^*$	$b^*- b_0^*$	ΔE
1	3.6 [5.99 ± 0.04]	450	3.5	-2.61 ± 0.32	-3.10 ± 0.26	-1.30 ± 0.06	-1.32 ± 0.09	1.65 ± 0.26	-0.49 ± 0.19	0.05 ± 0.14	1.74 ± 0.24
2	1.5 [6.43 ± 0.02]	511	1.4	-1.11 ± 0.12	-2.01 ± 0.01	-1.05 ± 0.10	-1.52 ± 0.02	1.40 ± 0.24	0.12 ± 0.09	-0.30 ± 0.09	1.44 ± 0.22
3	5.7 [5.72 ± 0.03]	511	1.4	-3.30 ± 0.08	-5.74 ± 0.13	-1.47 ± 0.01	-1.55 ± 0.02	0.97 ± 0.33	1.40 ± 0.04	0.19 ± 0.07	1.73 ± 0.21
4	1.5 [6.43 ± 0.02]	511	5.6	-3.91 ± 0.05	-4.06 ± 0.09	-2.36 ± 0.16	-2.77 ± 0.02	1.42 ± 0.24	-0.51 ± 0.10	-0.26 ± 0.09	1.54 ± 0.18
5	5.7 [5.72 ± 0.03]	511	5.6	-5.72 ± 0.15	-7.67 ± 1.77	-2.54 ± 0.01	-2.89 ± 0.25	0.94 ± 0.08	-0.66 ± 0.23	0.04 ± 0.06	1.16 ± 0.19
6	3.6 [6.01 ± 0.04]	600	0.0	-1.10 ± 0.04	-2.07 ± 0.12	-0.92 ± 0.26	-0.50 ± 0.13	0.14 ± 0.35	-0.61 ± 0.04	-0.49 ± 0.08	0.85 ± 0.05
7	0.0 [6.80 ± 0.03]	600	3.5	-4.44 ± 0.16	-5.39 ± 0.26	-2.26 ± 0.15	-3.79 ± 0.49	3.52 ± 0.14	-2.08 ± 0.17	-0.32 ± 0.08	4.11 ± 0.10
8	3.6 [6.01 ± 0.04]	600	3.5	-6.60 ± 0.24	-7.46 ± 0.24	-2.20 ± 0.21	-3.75 ± 0.35	1.30 ± 0.22	-0.96 ± 0.02	-0.02 ± 0.04	1.62 ± 0.17
9	3.6 [6.01 ± 0.04]	600	3.5	-6.49 ± 0.82	-7.06 ± 0.36	-2.16 ± 0.02	-4.22 ± 0.81	1.12 ± 0.73	-0.76 ± 0.08	-0.19 ± 0.14	1.44 ± 0.48
10	3.6 [5.99 ± 0.05]	600	3.5	-7.59 ± 0.32	-7.79 ± 0.24	-1.82 ± 0.33	-4.90 ± 0.85	1.86 ± 0.32	-0.20 ± 0.09	0.00 ± 0.14	1.88 ± 0.30
11	3.6 [5.99 ± 0.05]	600	3.5	-7.31 ± 0.23	-6.82 ± 0.13	-1.91 ± 0.11	-5.39 ± 0.00	1.81 ± 0.21	1.17 ± 0.06	0.05 ± 0.09	2.16 ± 0.20
12	3.6 [6.05 ± 0.05]	600	3.5	-6.18 ± 0.28	-6.31 ± 0.61	-1.98 ± 0.05	-5.40 ± 0.00	1.00 ± 0.46	-0.49 ± 0.04	0.44 ± 0.09	0.99 ± 0.32
13	3.6 [6.05 ± 0.05]	600	3.5	-6.49 ± 0.07	-7.97 ± 1.47	-1.83 ± 0.71	-5.40 ± 0.00	2.22 ± 0.39	-0.01 ± 0.13	0.67 ± 0.16	2.00 ± 0.41
14	3.6 [6.05 ± 0.05]	600	3.5	-6.49 ± 0.28	-6.35 ± 0.34	-1.98 ± 0.20	-5.33 ± 0.00	0.99 ± 0.08	-0.74 ± 0.08	0.17 ± 0.06	1.25 ± 0.10
15	3.6 [6.05 ± 0.05]	600	3.5	-6.16 ± 0.29	-6.35 ± 0.29	-1.85 ± 0.07	-4.99 ± 0.58	0.38 ± 0.23	-1.59 ± 0.11	0.11 ± 0.09	1.64 ± 0.14
16	3.6 [6.05 ± 0.05]	600	3.5	-6.62 ± 0.45	-6.42 ± 0.10	-1.94 ± 0.28	-4.99 ± 0.58	0.75 ± 0.34	-1.13 ± 0.14	0.35 ± 0.11	1.44 ± 0.12
17	7.2 [5.55 ± 0.05]	600	3.5	-6.83 ± 0.41	-7.55 ± 0.42	-2.31 ± 0.19	-5.02 ± 0.00	1.61 ± 0.10	1.26 ± 0.21	0.17 ± 0.08	2.05 ± 0.21
18	3.6 [5.99 ± 0.04]	600	7.0	-6.95 ± 0.11	-8.10 ± 1.36	-2.21 ± 0.19	-5.07 ± 0.58	2.41 ± 0.34	0.45 ± 0.11	0.68 ± 0.09	2.21 ± 0.36

Table 1. (continuation) *Salmonella,* aerobic mesophilic and lactic acid bacteria inactivation (log reduction) and changes in the colour lightness (*L**), redness (*a**) and yellowness (*b**) on raw pet food samples due to high pressure processing (HPP) treatments at each combination of the Central Composite Design.

Trial	Lactic acid (g/kg)[a]	Pressure (MPa)	Time (min)	Microbial inactivation (log reduction)[b]				Colour changes post HPP[c]			
				Salmonella		Mesophilic bacteria	Lactic acid bacteria				
				Post-HPP	Post-HPP + Frozen storage	Post-HPP	Post-HPP	$L^* - L_0^*$	$a^* - a_0^*$	$b^* - b_0^*$	ΔE
19	1.5 [6.43 ± 0.07]	689	1.4	-5.73 ± 0.13	-7.10 ± 0.37	-2.58 ± 0.28	-5.55 ± 0.00	3.43 ± 0.28	-1.20 ± 0.17	0.14 ± 0.09	3.64 ± 0.21
20	5.7 [5.66 ± 0.08]	689	1.4	-7.73 ± 0.00	-8.31 ± 1.16	-2.20 ± 0.24	-5.43 ± 0.00	0.96 ± 0.15	-1.97 ± 0.08	0.13 ± 0.02	2.20 ± 0.13
21	1.5 [6.43 ± 0.07]	689	5.6	-7.59 ± 0.35	-8.20 ± 1.36	-2.73 ± 0.06	-5.55 ± 0.00	4.82 ± 0.24	-0.14 ± 0.09	0.76 ± 0.03	4.88 ± 0.24
22	5.7 [5.67 ± 0.08]	689	5.6	-8.13 ± 1.46	-7.89 ± 1.54	-2.18 ± 0.03	-5.43 ± 0.00	2.08 ± 0.33	-0.95 ± 0.10	0.03 ± 0.05	2.29 ± 0.26
23	3.6 [6.09 ± 0.05]	750	3.5	-9.08 ± 0.94	-9.08 ± 0.94	-2.37 ± 0.18	-5.40 ± 0.00	2.91 ± 0.34	-0.27 ± 0.14	0.98 ± 0.15	2.76 ± 0.34

[a] Mean ± standard deviation of the pH of three replicates are reported between square brackets

[b] Mean ± standard deviation of three replicates.

[c] Mean ± standard deviation of three replicates. L_0^*, a_0^* and b_0^* indicate the measurements before the HPP treatment and L^*, a^* and b^* the measurements after the application of HPP on raw pet food. Delta E (ΔE) provides the insight into the difference seen betwee two colours.

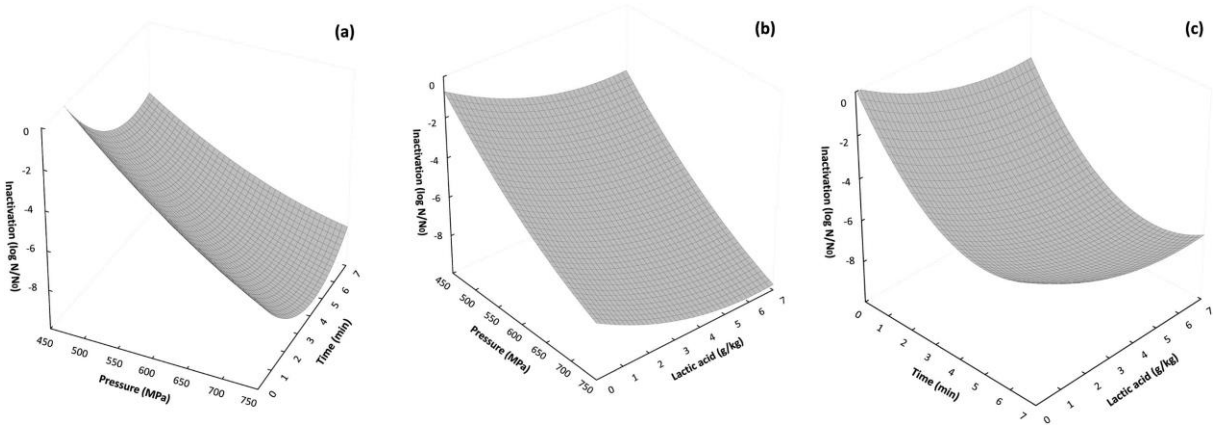


Figure 1. Response surface graphs of HPP-induced inactivation of *Salmonella* spp. in raw pet food according to the developed model: (a) pressure and holding time effects; (b) pressure and lactic acid effects; (c) holding time and lactic acid effects. The factors not included in each graph were maintained at the central value of the central composite design (lactic acid = 3.6 g/kg in graph (a), time = 3.5 min in graph (b), pressure = 600 MPa in graph (c)).

The coefficient of the quadratic term of lactic acid (Table 2, Eq.1), indicated that the enhancing HPP effect was stabilized at concentrations above 4.25 g/kg, as higher lactic acid concentration did not result in additional *Salmonella* inactivation at pressures equal or above 600 MPa (Figure 1a).

These results were relevant since the increase in the production costs due to the addition of higher amounts of lactic acid would not increase the safety of the product. This can be seen in Table 1 in the HPP treatment at 600 MPa for 3.5 min where *Salmonella* inactivation was 6.83 log in a product with 7.2 g/kg of lactic acid (Trial 17) compared to the mean of *Salmonella* inactivation (6.66 log) of the central points of the CCD with 3.6 g/kg of lactic acid (Trials 8-16). This difference was below 0.5 log and thus, was not relevant from a microbiological point of view. Since the industrial HPP equipment currently available can achieve maximum working pressures of 600 MPa, the addition of lactic acid in products intended to be pressurized products could be an effective strategy to enhance the level of safety and to ensure the compliance with current regulations for raw pet food concerning *Salmonella*.

3.2 Survival of Salmonella after HPP during subsequent frozen storage

The storage of pressurized samples at -18 °C for 14 days resulted in additional *Salmonella* inactivation, though the extent of further inactivation during the frozen storage varied depending on the HPP parameters and lactic acid concentration used in each the trial (Table 1). These results indicate that bacterial cells sub-lethally damaged by HPP were more susceptible to subsequent frozen storage. Similar results were reported for *E. coli* O157:H7 in ground beef submitted to HPP and subsequently frozen (Black et al., 2010; Zhou et al., 2016).

With the frozen storage, lactic acid exerted a quantitatively more noticeable effect throughout the tested range of lactic acid concentrations, being linear and interactive with pressure as shown by the polynomial model obtained (Table 2). Therefore, the presence of lactic acid, not only prevented the recovery of sub-lethally damaged cells after HPP, but also contributed to the loss of viability during the storage at -18 °C after HPP. These results were in accordance with the results reported by King et al. 2012 in which greater reductions of *Salmonella* of at least 1 log were observed in frozen-stored pork meat samples treated with lactic acid in comparison with those non-treated with the acid. Moreover, the interaction between lactic acid concentration and pressure (Table 2, Eq. 2) indicated that the enhancement of the lethality of the HPP effect by the lactic acid was dependent on the level of pressure. Therefore, and in accordance with the results reported in Section 3.1, the inactivation of *Salmonella* could be enhanced at lower pressure levels in acidulated products after the frozen storage, while at higher

pressure levels, the pressure would be sufficient to damage and inactivate *Salmonella* even without the addition of lactic acid.

Table 2. Results of the multivariate regression describing the effect of pressure, pressure-holding time and lactic acid concentration on the inactivation of *Salmonella* spp. (immediately after high pressure processing (HPP) and after 14 days of frozen storage), total aerobic mesophilic bacteria, lactic acid bacteria and lightness in raw pet food.

Microorganism / colour parameter	Treatment	Model[a]	RMSE
Salmonella spp.	HPP	$\text{Log}\,(N/No) = 27.92 - 0.06217 \cdot P - 3.3580 \cdot t - 1.051 \cdot LA + 0.00003 \cdot P^2 + 0.2013 \cdot t^2 + 0.06628 \cdot LA^2 + 0.00193 \cdot (P \cdot t) + 0.05765 \cdot (t \cdot LA)$ Eq. (1)	0.635
	HPP + frozen storage	$\text{Log}\,(N/No) = 22.9733 - 0.04152 \cdot P - 2.7381 \cdot t - 2.9986 \cdot LA + 0.1251 \cdot t^2 + 0.0022 \cdot (P \cdot t) + 0.0043 \cdot (P \cdot LA)$ Eq. (2)	1.021
Mesophilic bacteria	HPP	$\text{Log}\,(N/No) = 5.818 - 0.0123 \cdot P - 1.2350 \cdot t - 0.3692 \cdot LA + 0.0231 \cdot t^2 - 0.0331 \cdot LA^2 + 0.0015 \cdot (P \cdot t) + 0.0010 \cdot (P \cdot LA)$ Eq. (3)	0.208
Lactic acid bacteria	HPP	$\text{Log}\,(N/No) = 33.21 - 0.0936 \cdot P - 2.5040 \cdot t + 0.00006 \cdot P^2 + 0.1557 \cdot t^2 + 0.0017 \cdot (P \cdot t)$ Eq. (4)	0.702
Lightness (L^*)	HPP	$L^* - L_0{}^* = 10.99 - 0.0393 \cdot P - 0.7910 \cdot t + 0.7056 \cdot LA + 0.00004 \cdot P^2 + 0.0971 \cdot LA^2 + 0.0017 \cdot (P \cdot t) - 0.0029 \cdot (P \cdot LA)$ Eq. (5)	0.507

[a]Where $Log\,(N/No)$ is the bacterial inactivation, *P* is pressure level, *t* is the holding time, *LA* is lactic acid and $L^* - L_0{}^*$ is the difference in product lightness due to the HPP treatment.

In a previous work (Serra-Castelló et al., 2022) dealing with the kinetics of HPP inactivation of *Salmonella* in raw pet food, the storage under refrigeration of raw pet food after HPP allowed the recovery of sublethally injured cells, though the addition of lactic acid minimised the recovery. As a result, about 2-log higher levels of *Salmonella* could be counted when samples were analysed after being stored for 24h at 4 °C after being pressurised at 600 MPa for 7 min. On the contrary, the results of the present work indicate that the storage of raw pet food intended for cat (formulated without and with lactic acid) stored under frozen conditions after the HPP treatment could be a feasible and effective control measure applied by manufacturers to avoid the recovery of sublethally-injured *Salmonella* cells.

3.3 Endogenous microbiota of raw pet food

Counts of endogenous microbiota in non-inoculated raw pet food samples before HPP were 2.81 ± 0.73 log cfu/g for *Enterobacteriaceae*, 2.16 ± 0.45 log cfu/g for *Pseudomonas* spp., 4.63 ± 0.20 log cfu/g for total aerobic mesophilic bacteria and 4.34 ± 0.19 log cfu/g for LAB. Due to the relatively low initial levels of *Enterobacteriaceae* and *Pseudomonas* spp., the HPP effect on both groups could not be evaluated as they were reduced to levels below the plate detection limit in the majority of the trials performed (data not shown). Argyri et al. (2019) reported that the HPP treatment of chicken fillets at 500 MPa for 10 min resulted in a reduction of the inoculated *Enterobacteriaceae* and *Pseudomonas* levels of approximately 6 log, indicating the high susceptibility of both bacterial groups to HPP.

Total aerobic mesophilic bacteria showed to be the bacterial group less affected by HPP treatments (Table 1). Reductions from 0.92 to 2.73 log were recorded in the different trials (Table 1). The inactivation of total aerobic mesophilic microorganisms depended on the three technological parameters studied, i.e., pressure, holding time and concentration of lactic acid (Table 2, Eq. 3). The pressure exerted a linear effect, though the interaction with holding time and with the concentration of lactic acid reflected that pressure also modulated the effect of these two parameters. Thus, inactivation increased almost linearly over time, with the appearance of slight resistance tail at the central pressure value of the CCD (around 600 MPa). The effect of the lactic acid was statistically significant, so it was included in the mathematical model. However, from the microbiological perspective, in most

of the trials it was hardly relevant because differences in inactivation between concentrations of lactic acid in treatments below 600 MPa and/or 5 min were < 0.5 log units. On the other hand, the significance of the quadratic and interaction terms with pressure was surprising, indicating that pressure levels above 600 MPa lead to lower inactivation levels in the presence of increasing amounts of lactic acid. Among the plausible hypotheses that could explain these results, there is the fact that the group of total aerobic mesophilic microorganisms is formed by a great variety of genera, species (including sporulated bacteria) and strains with different resistance against the studied factors.

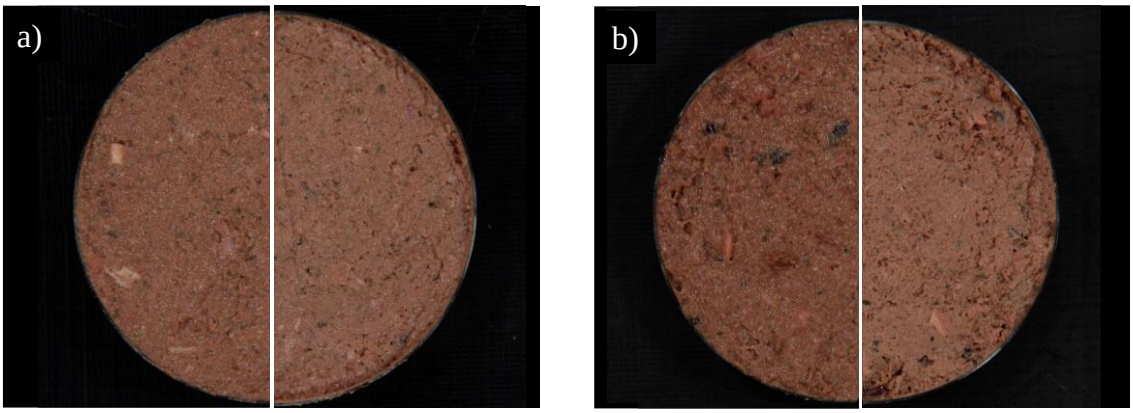
For LAB, the significant and relevant factors determining the HPP inactivation were pressure and holding time (Table 2, Eq. 4). The pressure had a linear effect on LAB inactivation, being the impact of the quadratic factor not significant. At higher pressures (> 600 MPa), the levels of lactic acid bacteria were below the quantification limit in most of the trials. In contrast, the quadratic effect of the holding time was more pronounced and would describe the maximum inactivation values that could be quantified taking into account the initial levels of LAB. The effect of lactic acid was not significant, which can be explained by the fact that LAB are relatively tolerant to this acid as it is a product of their own metabolism.

3.4 HPP effect on raw pet food colour

The results of the evaluation of the instrumental colour of raw pet food on non-inoculated samples subjected to different HPP treatments according to the CCD are shown in Table 1. The HPP caused a slight decrease of redness (a^*) in most of the samples, while the yellowness (b^*) parameter generally slightly increased (Table 1). Nevertheless, when fitting models to colour data measurements, a lack of fit was obtained for a^* and b^* parameters, indicating that neither the pressure level, holding time or lactic acid contributed to explain the slight differences in redness and yellowness showed by the HPP-treated product.

An increase on the parameter L^* was detected, which means that the pressurized samples presented a slightly lighter (white) than the non-pressurized, which can be attributed to the denaturation of myofibrillar proteins (Kruk et al., 2011). These results are in accordance with published studies showing an increase in the L^* parameter after the pressurization of poultry meat (Yuste et al., 1999; Beltran et al., 2004; Mariutti et al., 2008; Del Olmo et al., 2010; Kruk et al., 2011; Omana et al., 2011; Cap et al., 2020).

Modelling the lightness (L^*) resulted in quadratic terms for pressure and time (Table 1, Eq. 5) indicating that the impact of these factors on the lightness of the matrix was evidenced in a relevant way from a certain level of pressure (around 600 MPa) and treatment time (approximately 5 min). The presence of increasing concentrations of lactic acid would have a seemingly protective effect of the change in lightness, since for a certain level of pressure and/or treatment time the difference in lightness (L^*-L_0^*) before and after the treatment was reduced. This could be explained by the fact that the lightness value used to calculate the difference was determined in the product matrix once the lactic acid was incorporated.



Colour parameter	No-HPP	Post-HPP
L^1	45.81	47.46
a^2	13.68	13.19
b^3	11.69	11.74

Colour parameter	No-HPP	Post-HPP
L^1	43.17	47.99
a^2	14.43	14.29
b^3	11.39	12.15

Figure 2. Visual colour appearance of raw pet food formulated with lactic acid before (No-HPP) and after pressurization (Post-HPP) at (a) 450 MPa for 3.5 min (with 3.6 g/kg lactic acid, Trial 1) and at (b) 750 MPa for 3.5 min (with 1.5 g/kg lactic acid, Trial 21).

The total colour change (ΔE) was below 3 in most of the trials, except in trial 7 (without lactic acid), trial 21 (low lactic acid concentration and pressure level) and trial 23 (highest pressure level), showing ΔE higher than 3. The slight change of the lightness of the matrix when measured instrumentally was not perceived as a drawback from the commercial point of view as the visual colour appearance of the HPP product (Figure 2, comparing the product from trials with low and high ΔE) was considered to be within the reasonably foreseeable range of variability among production batches.

4 Conclusions

High pressure processing points out as a strategy that can be applied by manufacturers of chicken-based raw pet food as a technological measure to inactivate pathogenic and non-pathogenic bacteria without causing relevant negative effects in the appearance of the product. The formulation of chicken-based raw pet food with lactic acid as well as the subsequent frozen storage of pressurised products enhances the HPP lethal effects avoiding the recovery of pressure-injured cells during storage of chicken-based raw pet food and, in addition, promoted a further inactivation of *Salmonella*. The predictive models developed in this study can constitute a useful decision tool to help manufacturers of chicken-based raw pet food to increase the microbiological safety of their products by allowing the selection of most effective pressure level, holding time and lactic acid combinations to achieve target levels of bacterial inactivation.

5 Acknowledgements

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6 Declaration of conflict of interests

Authors declare no conflict of interest. The funders provided the raw materials for preparing the raw pet food product used in the study. They had no responsibility on the design of experiments, data collection and analysis or decision to publish.

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Article 4

Modelling the piezo-protection effect exerted by lactate on the high pressure resistance of *Listeria monocytogenes* in cooked ham

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Modelling the piezo-protection effect exerted by lactate on the high pressure resistance of *Listeria monocytogenes* in cooked ham

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Abstract

Food safety is often based on the application of several preservative (hurdle) factors whose combination must be smartly selected. The aim of the present study was to evaluate the effect of lactate and diacetate on the high pressure processing (HPP) inactivation of three *L. monocytogenes* strains (CTC1011, CTC1034 and Scott A) in sliced cooked ham. Inoculated vacuum-packed slices of cooked ham formulated without organic acids and with lactate, diacetate or the combination of both were pressurized at 400 MPa for different holding times and the inactivation kinetics were characterised by fitting primary and secondary models. The shape of the inactivation curves for *L. monocytogenes* depended on both product formulation and strain. Interestingly, lactate caused a dose-dependent piezo-protection in all three strains, as the HPP inactivation rate decreased in cooked ham formulated with increasing amounts of lactate and in comparison with the control product. The design, validation and implementation of HPP requires a tailor-made approach, considering product formulation and selection of strain/s.

Keywords:

High hydrostatic pressure; Pressurization; Food Safety; Meat Products; Pathogens; Organic Acids.

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1 Introduction

Listeria monocytogenes is a foodborne pathogen that can cause listeriosis, a severe human illness often associated with the consumption of ready-to-eat (RTE) products, particularly those favoring the growth of the pathogen during the refrigerated storage. Among RTE food, cooked meat products commercialized in a convenient format (i.e. sliced, diced, and packaged) are particularly considered of high risk according to the risk assessments developed by several organizations worldwide (EFSA BIOHAZ Panel, 2018; FDA/USDA, 2013).

Food safety criteria regarding *L. monocytogenes* in RTE products differ between countries, *e.g.* EU and USA. Regulation (EC) 2073/2005 (European Commission, 2005) establishes a maximum of 100 cfu/g of *L. monocytogenes* during the shelf-life of the product, whereas in USA a zero-tolerance policy is imposed (FSIS, 2003), which means the presence of the pathogen is not allowed either in product or on food contact surfaces. The zero-tolerance poses a challenge for the meat industry to comply with such regulation due to the technical difficulties for the control and complete eradication of *L. monocytogenes*.

To accomplish with the microbiological criteria for *L. monocytogenes* in RTE food control measures can be implemented. The purpose of these measures is (i) to minimize the occurrence of the pathogen in raw materials, (ii) to reduce its levels by applying lethality or post-lethality treatments (PLT) and/or (iii) to prevent its increase (either by recontamination or growth) through the use of antimicrobial agents (AMA) or processes, among others. In this framework, there are regulations such as those of the USA and Canada (FSIS, 2003; Health Canada, 2011) that follow a risk-based approach to identify alternative operating methods for an effective control of *L. monocytogenes* in post-lethality exposed RTE products, classifying the manufacturers according to the risk associated with their products. In the USA, the Listeria Rule establishes that the safest operating procedures are those validated as alternative 1, which rely on the combination of alternatives 2a and 2b. Alternative 2 consists in the application of a PLT to reduce or eliminate the contamination (Alternative 2a), or an AMA to reduce or inhibit the growth of *L. monocytogenes* (Alternative 2b, considered of higher risk than alternative 2a). The higher risk occurs when operation procedures rely exclusively on sanitation and good manufacturing practices (i.e. Alternative 3) (FSIS, 2003).

High pressure processing (HPP) is a non-thermal technology usually used as in-package PLT particularly interesting for products exposed to microbial contamination after the lethality treatment (i.e. during slicing and packaging operations). HPP is a widespread application in the meat industry. The microbial inactivation during HPP depends on technological factors (pressure, time and temperature) as well as food intrinsic factors (e.g., pH, a_w and food preservatives), either by favouring *L. monocytogenes* inactivation in case of low pH, or by exerting a protective effect in case of low a_w (Hereu, Dalgaard, Garriga, Aymerich, & Bover-Cid, 2012; Rendueles, Omer, Alvseike, Alonso-Calleja, Capita, & Prieto, 2011). Due to this product-specific lethal effect of HPP, the application of HPP as a PLT of RTE food products must be validated to reduce at least 1 log of *L. monocytogenes* in the product and an increased level of control is considered when a 2 log reduction of *L. monocytogenes* is documented (FSIS, 2014, 2015).

Among AMA, organic acids and their salts (e.g., lactate, diacetate) are frequently used by the meat industry within a natural biopreservation strategy (Pérez-Rodríguez, Carrasco, Bover-Cid, Jofré, & Valero, 2017), with levels varying from 1.5 to 3.0% of lactate, added alone or together with diacetate at levels from 0.10 to 0.25% (Mbandi & Shelef, 2001; Mellefont & Ross, 2007a; Porto-Fett et al., 2010). The efficacy of an AMA mainly depends on the type and amount of antimicrobial added and its mode of application (Aymerich, Garriga, Jofré, Martín, & Monfort, 2006). AMA should limit the growth of *L. monocytogenes* over the shelf-life of the product and they must be validated to allow no more than 2 log growth of *L. monocytogenes* (FSIS, 2014, 2015). In the framework of the implementation of the EU microbiological criteria (European Commission, 2005), a product (e.g. formulated with an AMA) belongs to the RTE food category that does not support the growth of *L. monocytogenes* when no more than 0.5 log units of pathogen growth is observed throughout the product shelf-life (EURL Lm, 2019). Several studies report the survival and growth capacity of *L. monocytogenes* in the presence of AMA, especially organic acids and their salts (Bover-Cid et

al., 2019; Mellefont & Ross, 2007b). However, the interaction between HPP and organic acids or their salts has been scarcely studied. According to the hurdle technology (Leistner, 2000), an increased effectiveness in controlling *L. monocytogenes* survival/growth (synergistic or additive effect) should be expected with the intelligent combination of hurdles. Despite this, data available in literature suggested that the addition of lactate in meat products, such as cooked ham and dry-cured ham, increases the HPP resistance of *L. monocytogenes* causing a piezo-protection that reduces the efficacy of the HPP (Table 1).

Table 1. High pressure inactivation of *L. monocytogenes* in meat products formulated without and with lactate reported in literature.

RTE product	*L. monocytogenes* strain	% Lactate (mode of application)[a]	HPP Treatment	HPP INACTIVATION (log reduction)			Reference
				Control (no lactate)	With Lactate	Difference (control-lactate)	
Cooked ham	Cocktail (CTC1010, CTC1011 and CTC1034)	1.40 (MB)	400MPa, 10 min	3.10	2.51	*0.59*	Marcos et al. (2008)
		1.80 (MB)	400MPa, 10 min	0.66	0.59	*0.07*	Aymerich et al. (2005)
		1.80 (IL)	400MPa, 10 min	1.76	1.50	*0.26*	Jofré et al. (2007)
		1.80 (MB)	600MPa, 5 min	3.79	3.71	*0.08*	Jofré et al. (2008)
Dry-cured ham	Cocktail (CECT4031, CTC1011 and CTC1034)	2.60 (B)	600MPa, 5 min	1.10	0.80	*0.30*	Stollewerk et al. (2012)
		2.60 (B)	600MPa, 5 min	1.60	0.22	*1.38*	Stollewerk et al. (2014)
Cooked turkey	Non-specified	1.80 (MB)	350MPa, 2 min	0.85	0.54	*0.31*	Lerasle et al. (2014)
		1.80 (MB)	350MPa, 8 min	1.42	0.81	*0.61*	Lerasle et al. (2014)
		1.80 (MB)	350MPa, 14 min	1.96	1.20	*0.76*	Lerasle et al. (2014)

[a]: Mode of application: B: during salting step; IL: active packaging (surface); MB: meat batter (additive in the product formulation)

In this framework, the present study aimed to evaluate the potential piezo-protective effect of organic acid salts used as AMA to formulate cooked meat products treated by HPP. A modelling approach was applied in order to quantitatively characterize the HPP-inactivation kinetics of three different *L. monocytogenes* strains inoculated on cooked ham formulated without or with natural antimicrobials often used by the meat industry, i.e. potassium lactate (food additive EU code: E-326) and sodium diacetate (E-262) and thus, to quantify the potential piezo-protective effect of organic acid salts towards *L. monocytogenes* HPP-inactivation in cooked ham.

2 Material and methods

2.1 Preparation of cooked ham

Cooked ham was prepared as in previous works (Bover-Cid et al, 2019; Hereu et al., 2012) with pork shoulder minced in a cutter to a particular size of 4 mm, and the following additives (g/Kg): water, 115; salt, 20.7; dextrose, 5.8; sodium tri-polyphosphate, 5.8; carrageenan, 2.3; $NaNO_2$, 0.1 and L-ascorbate, 0.6. Five batches of cooked ham were prepared by adding different types and amounts of organic acid salts, and consisted of:

i. 1.4 % of potassium lactate corresponding to 2.4% of HiPure product (Corbion®, Montmeló, Spain) added in the product formulation;
ii. 2.8% of potassium lactate corresponding to 4.7% of HiPure product (Corbion®, Montmeló, Spain) added in the product formulation;
iii. 0.1% of sodium diacetate (Grama Aliment SL, Les Preses, Spain);
iv. 1.4% potassium lactate and 0.1% sodium diacetate corresponding to 2.5% of Optiform (Corbion®, Montmeló, Spain) added in the product formulation;
v. a control batch was prepared without the addition of organic acids.

Though the addition of lactate and/or diacetate may influence the sensory characteristics of the product, the type and the amount of organic acid salts studied in the present work are within the ranges applied by the meat industry, thus resulting in products with sensory characteristics accepted by the consumers (Mellefont & Ross, 2007a; Porto-Fett et al., 2010).
Ingredients were homogenized in a mixer (model 35P, Tecnotrip S.A., Terrassa, Spain) for 30 min and stuffed using a stuffing machine (model H15, Tecnotrip S.A., Terrassa, Spain) into impermeable plastic film (Prolan SV 150, PHH, San Boi de Llobregat, Spain). The product was cooked in an oven at 75 °C until internal temperature reached 72 °C (total cooking time *ca*. 2.6 h).
In agreement with previous reports (Mellefont & Ross, 2007a), the addition of lactate and/or diacetate did not significantly change the physicochemical parameters of cooked ham compared to the control batch and the manufactured product had a pH of 6.04 ± 0.04 and a_w of 0.974 ± 0.003.

2.2 Inoculation of L. monocytogenes in sliced cooked ham and HPP

L. monocytogenes strains used in this study were the strains CTC1011 (serotype 1/2c) and CTC1034 (serotype 4b) both isolated from meat products and belonging to the Institute of Agriculture and Food Research and Technology (IRTA)-Food Safety Program's collection, as well as the strain Scott A (serotype 4b), a clinical isolate frequently included in HPP inactivation studies (van Boeijen, Moezelaar, Abee, & Zwietering, 2008). Cultures were prepared by growing each strain in Brain Heart Infusion (BHI) broth (Beckton Dickinson, Sparks, Md., USA) at 37 °C for 7 h and subsequently at 37 °C for 18 h (i.e. till the stationary phase of growth was reached) in two consecutive subcultures. Final cultures were preserved frozen at −80 °C in the growth medium supplemented with 20% glycerol until their use. Freezing conditions expose cells to concentrated solutes, which cause an osmotic stress similar to that caused by dry environments occurring in the food industry (e.g. clean and dry food contact surfaces). Additionally, some industrial processes to prepare sliced RTE products include a pre-freezing step to facilitate the slicing process (Hereu et al., 2012; Hereu, Dalgaard, Garriga, Aymerich, & Bover-Cid, 2014).
Cooked ham was sliced aseptically in slices of 12-14 g (1.5 mm thick). The frozen cultures thawed at room temperature were used to independently inoculate each strain at 1% v/w to achieve *ca*. 10^7 CFU/g. The inoculum was spread on the surface of the slices with a sterile spreader until absorbed (<1 min in a biosafety cabinet).

Inoculated slices were individually vacuum-packaged (EV-15-2-CD; Tecnotrip, Terrassa, Spain) in plastic bags of PET/PE (oxygen permeability <50 cm^3/m^2/24 h and water vapor permeability <15 mg/m^2/24 h; Sacoliva S.L., Barcelona, Spain).
HPP was performed in a Wave6000/120 industrial equipment (Hiperbaric, Burgos, Spain) at 400 MPa and holding times of 0, 2.5, 3.75, 5, 6.25, 7.5, 8.5, 9.5 and 10 min. According to the data recorded through the SCADA system of the HPP equipment, the come-up time was 2.0 min and the pressure release time was almost immediate (<2s). The pressurization fluid was water, and the initial temperature was set at 13 °C. After pressurization, the samples were kept for 2 h at room temperature before *L. monocytogenes* analysis. The HPP treatments applied to cooked ham are known to have no or minimal impact on the physico-chemical and sensory characteristics of cooked meat products (e.g. Hereu et al., 2012; Olmo, Calzada, & Nuñez, 2014; Vercammen et al., 2011), which was confirmed by the visual observation of samples before the microbiological analysis (data not shown).

2.3 Microbiological analysis

Each sample (12-14 g) was aseptically minced, 1/10 diluted in Tryptic Soy Broth (Becton, Dickinson) supplemented with 0.6% yeast extract (TSBYE) and homogenized for 1 min in a bag blender (Smasher, Biomerieux, France). Samples were kept at room temperature for 1 hour following the ISO recommendations before preparing the appropriate serial dilutions in 0.1% Bacto Peptone (Difco Laboratories, Detroit, MI, USA) with 0.85% NaCl. Samples were then spread plated on Chromogenic Listeria Agar (Oxoid Ltd., Basingstoke, UK) and incubated at 37 °C for 48 h. Duplicate or triplicate analysis for each batch and strain was performed.
The presence/absence of the pathogen was investigated in samples with expected concentration of *L. monocytogenes* below the quantification limit. Ten-g samples were 1/10 diluted in TSBYE, homogenized and incubated at 37 °C for 48 h. After enrichment, the presence of *L. monocytogenes* was investigated by plating on Chromogenic Listeria Agar (Hereu et al., 2012; 2014). For modelling purposes, presence below the quantification limit was assumed as 1 cfu/g and absence as 0.1 cfu/g.
The absence of detectable levels of spoilage specific organisms (i.e. lactic acid bacteria) in cooked ham slices was checked by plating 1 ml of the homogenized 1/10 dilution into MRS (de Man, Rogosa and Sharpe) agar plates (Merck KGaA, Darmstadt, Germany), which were incubated at 30 °C for 72 h under anaerobiosis.

2.4 Data analysis

L. monocytogenes counts were log transformed and the pathogen inactivation calculated as log N/N_0. To estimate the kinetic inactivation parameters, the primary inactivation Weibull model (Eq. 1) was fitted to the inactivation data (log N/N_0) along HPP holding time, using the nls2 and nls functions from the respective nls2 and nls packages of R (R Core Team, 2013).

$$\log N/N_0 = (\log N/N_0)_i - \left(\frac{t}{\delta}\right)^p \qquad \text{Eq. 1}$$

where $(\log N/N_0)_i$ is a fixed value representing the average value of the initial bacterial inactivation of 3 replicates at t = 0 (i.e. a cycle of pressure come-up and release without holding time), δ is the holding time (min) required for the first log reduction, p is a dimensionless parameter describing the shape of the inactivation curve and t is the holding time (min) during HPP. The characteristics of the Weibull model in terms of flexibility (being able to fit most typical survivor curves depending on the p parameter, i.e. p<1 concave; p=1 linear and p>1 convex), its parsimony and meaningfulness of the δ parameter (i.e. close to widely known decimal reduction time, D) to be used in secondary modelling are the reasons reported to

recommend this model for fitting microbial inactivation curves associated with food processing and preservation treatments (van Boekel, 2002).

To quantitatively characterize the effect of lactate on the kinetic inactivation parameters (δ and p), polynomial models were developed for each strain. The fit using different transformations of kinetic parameter estimates (δ and p), including square root, inverse, ln and log were assessed. Stepwise regression was carried out to obtain equations with only the significant parameters using R software (R Core Team, 2013).

Besides the classical two-step modelling approach described above, the one-step or global regression procedure was applied by integrating the primary Weibull model into the polynomial secondary models (Eq. 2) for the kinetic inactivation parameters and fitting it to the entire data set of inactivation values (n=225) for HPP cooked ham formulated without and with different concentrations of potassium lactate.

$$\log N/N_0 = (\log N/N_0)_i - \left(\frac{t}{(a+b*LAC^2)}\right)^p \qquad \text{Eq. 2}$$

where $(\log N/N_0)_i$ is a fixed value representing the average value of the initial bacterial inactivation of 3 replicates at t = 0, t is the holding time (min), p is a dimensionless parameter describing the shape of the inactivation curve (i.e. p<1 concave; p=1 linear and p>1 convex) and *lactate* is the concentration of potassium lactate added (%). The parameters a and b are the coefficients estimates of the regression describing the effect of lactate (*LAC*) on the time for the first log reduction (δ).

The statistical goodness of fit of the primary models was assessed by means of residual sum of squares (RSS) and root mean of square error (RMSE). The RSS was derived by summing the squared differences between the experimental (observed) data and the value provided by the model (fitted data). The RMSE was calculated as the square root of the Mean Sum of Squared Errors (MSSE), which were derived by dividing the RSS by the number of degrees of freedom (i.e. the number of data points minus the number of parameters and initial values used). For the secondary models the adjusted determination coefficient (R^2_{adj}) as in Eq. 3 was also considered (Spiess & Neumeyer, 2010). The F-test was applied to assess the need of different models for the three *L. monocytogenes* strains studied (Zwietering, Jongenburger, Rombouts, & van't Riet, 1990).

$$\mathrm{R}^2_{\mathrm{adj}} = \frac{(n-1)\cdot R^2 - k + 1}{n-k}$$

Where R^2 is the coefficient of determination, i.e. 1−RSS/SSTO, with SSTO being the sum of the squared differences between the experimental (observed) values and the mean of these experimental values.

3 Results and discussion

3.1 L. monocytogenes behaviour in pressurized cooked ham without organic acids

The high pressure inactivation kinetics of the 3 tested strains of *L. monocytogenes* in cooked ham formulated without organic acids are shown in Figure 1 (a,b,c) with fitted kinetic parameters of the Weibull model shown in Table 2. As expected, inactivation of *L. monocytogenes* was higher as the holding time increased from 0 to 10 min. However, inactivation curves of different shape were found for the different strains. *L. monocytogenes* CTC1011 showed a convex shape with a considerable shoulder described by a δ value, i.e. the holding time needed for the first log reduction, of almost 6 min. The pronounced shape of the inactivation curve (p >3) observed for holding times higher than 6 min was due to the virtually total inactivation of the inoculated pathogen (not detected) in some samples. At higher holding times (> 6 min), the inactivation of CTC1011 was 3 log higher than CTC1034 and Scott A. Inactivation of CTC1034 followed a linear curve trend, with a p parameter close to 1, resulting in almost constant effect of HPP in the

inactivation kinetics across 10 min of holding time. The δ found for CTC1034 had a value close to 4 min, indicating that CTC1034 was more sensitive to HPP at lower holding times than CTC1011.

The concave shape for the inactivation curve (p<0.5) of the clinical isolate *L. monocytogenes* Scott A resulted in much lower holding time to achieve the first log reduction (δ = 0.7 min) compared to CTC1011 and CTC1034. However, the shape was compatible with the occurrence of a resistant tail for holding times higher than 6 min. Thus, Scott A was the most sensitive strain to HPP at lower holding times but also the most resistant to HPP at higher times.

Table 2. Estimated kinetic parameters resulting from fitting the Weibull model to *L. monocytogenes* inactivation data (log N/N_0) on different formulations of cooked ham pressurized at 400 MPa.

Experimental conditions			Kinetic parameters[a]			Goodness of fit[b]	
Added lactate (%)	Added diacetate (%)	*L. monocytogenes* strain	$(\log N/N_0)_i$	δ (min)	p	RSS	RMSE
-	-	CTC1011	0.03	5.98	3.62	5.691	0.497
-	-	CTC1034	-0.14	3.89	1.29	6.113	0.516
-	-	Scott A	-0.32	0.70	0.47	19.669	0.946
1.40	-	CTC1011	-0.09	7.00	5.04	11.131	0.696
1.40	-	CTC1034	-0.10	4.62	1.43	28.949	1.122
1.40	-	Scott A	-0.09	0.85	0.40	11.099	0.695
2.80	-	CTC1011	-0.10	7.39	4.48	6.561	0.534
2.80	-	CTC1034	-0.18	7.48	1.17	3.991	0.417
2.80	-	Scott A	-0.13	2.48	0.41	7.961	0.588
-	0.10	CTC1011	-0.18	5.21	2.95	12.884	0.748
-	0.10	CTC1034	-0.12	2.67	1.01	18.786	0.904
-	0.10	Scott A	-0.35	0.56	0.45	30.172	1.145
1.40	0.10	CTC1011	-0.10	6.34	4.02	11.839	0.717
1.40	0.10	CTC1034	-0.10	3.97	1.08	6.619	0.536
1.40	0.10	Scott A	-0.32	1.28	0.51	12.914	0.749

[a] $(\log N/N_0)i$ is the average value of the initial bacterial inactivation of 3 replicates at t = 0, δ: holding time for the first log reduction; p: shape of the inactivation curve

[b] n=25 data points (log N/N_0) of each combination of conditions were included for fitting. RSS: residual sum of squares; RMSE: root mean squared error; R^2_{adj}: adjusted coefficient of determination.

The need of different holding times to achieve the first log reduction (δ = 0.5-6 min) and the differences in the shape (concave, linear, convex) proved that inactivation curves, and thus, their piezo-resistance, were highly dependent on the *L. monocytogenes* strain. The strain-specific resistance to HPP could be related with the strain membrane composition and properties to withstand pressure (Jung, Lee, Lee, Kim, & Ahn, 2013).

To the best of the author's knowledge, few studies are available describing the impact of HPP on the *L. monocytogenes* membrane. Although that, for gram-negative bacteria it has been shown that the bacterial membrane integrity is often compromised with the application of HPP, leading to morphological and physiological changes (Ma et al., 2019). Within this framework, Klotz et al. (2010) hypothesized that the differences in pressure resistance observed between two strains of *E. coli* in the range of 100 to 700 MPa were related to the dissimilar ability of their membranes to withstand pressure. More specifically, for some *Salmonella* strains, Tamber (2018) found that the higher the proportion of cyclopropane fatty acids in the bacterial membrane the higher the resistance to HPP. These results were in agreement with those reported by Charoenwong et al. (2011) in which the cyclopropane fatty acid synthase had a decisive role on the HPP resistance of *E. coli*. On the other hand, HPP was shown to induce an elongation of

L. monocytogenes cells, leading to an increased permeability of the membrane and to a rupture of the internal cellular structure (Jung et al., 2013). In this line, the degree of pressure resistance has been related with the ability of the cells to repair ion leaks of the membrane (Na^+, K^+, Mg^{2+} and Ca^{2+}) after decompression (Farkas & Hoover, 2000; Ma et al., 2019).

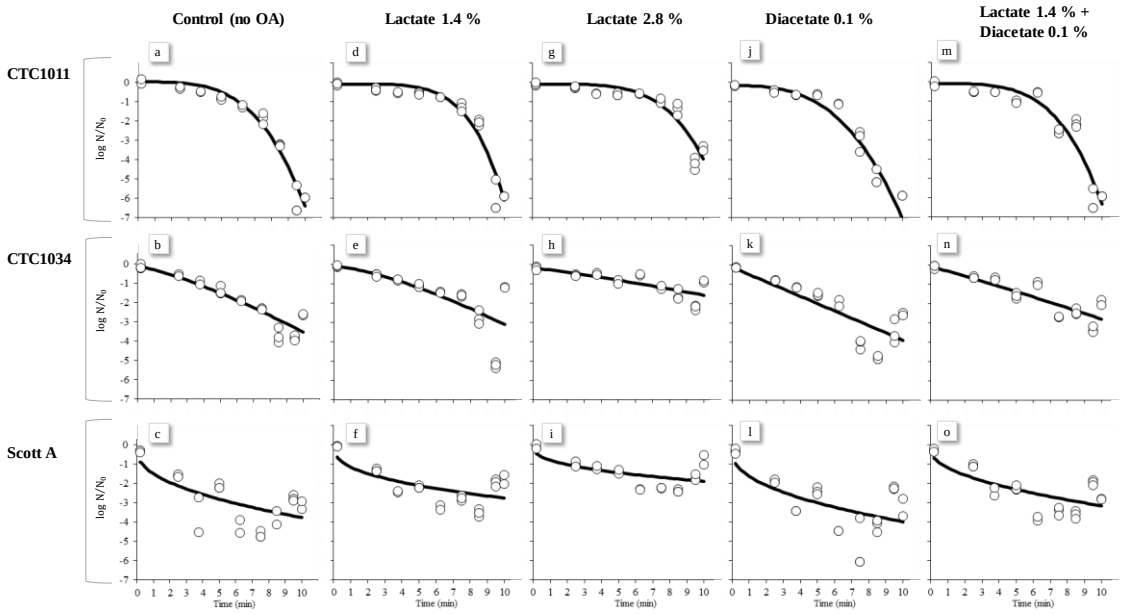

Figure 1. Inactivation of *L. monocytogenes* strains (CTC1011, CTC1034 and Scott A) in cooked ham formulated without (control) and with organic acids (OA) pressurized at 400 MPa for different holding times. Symbols represent the experimental observed inactivation ($\log N/N_0$) data and lines the fit of the Weibull model.

Besides this, some authors showed that the genetic features of each strain play a role in *L. monocytogenes* HPP resistance. Karatzas et al. (2003) reported that some piezo-tolerant isolates of *L. monocytogenes* had a mutation in the CtsR gene, leading to a loss of its function and to an increased expression of Clp proteases (which prevent harmful accumulation of damaged proteins) that confer resistance of *L. monocytogenes* cells to HPP. Moreover, the resistance to HPP observed for some *L. monocytogenes* strains was not related with mutation in the CtsR gene, which suggest that other mechanisms may confer resistance to HPP such as proteins involved in stress responses which are mainly regulated by the RpoS gene (Karatzas, Valdramidis, & Wells-Bennik, 2005; Landini, Egli, Wolf, & Lacour, 2014; Chen, Neetoo, Ye, & Joerger, 2009; Gayán, Cambré, Michiels, & Aertsen, 2017; Gayán, Rutten, Van Impe, Michiels, & Aertsen, 2019)
Some authors also described the importance of strain ability to accumulate compatible solutes to withstand pressure, such as a proline, whose synthesis is strain-dependent and conditioned by the food matrix components (Bartlett, 2002; Considine, Sleator, Kelly, Fitzgerald, & Hill, 2011).
Overall, the different mechanisms described above involved in *L. monocytogenes* resistance to HPP could lead to a different degree of pressure resistance depending on the temporal frame along the HPP; such strain specific resistance can explain the different shapes shown by the inactivation curves.

3.2 L. monocytogenes behaviour in pressurized cooked ham with organic acids

The presence of salts of organic acids in the cooked meat products did not modify the shape (convex, linear or concave) of the inactivation curves of the *L. monocytogenes* strains compared to those found in cooked ham without organic acids (Figure 1). However, the extent of the inactivation and the corresponding inactivation kinetic parameter values differed depending on the type and concentration of added organic acid salt (Table 2).
The addition of lactate increased the HPP resistance of all the strains. In all cases, the inactivation was lower than in control products and the inactivation kinetic curve moved upwards (Table 2, Figure 1 d, e, f, g, h, i).

This fact empirically confirms that lactate exerts a piezo-protective effect on *L. monocytogenes* inactivation in cooked ham in a strain and dose-dependent magnitude. In addition, this finding is in accordance with previous studies in which HPP was systematically reported to be less effective when lactate was used as antimicrobial in the formulation of meat products or the packaging (Table 1).

At the maximum holding time assessed (10 min), inactivation of *L. monocytogenes* strains in control batches (without organic acids) was higher than in products with 1.4% of added lactate. At this holding time, the difference in inactivation in observed values when comparing control and 1.4% lactate ham was of 0.5, 1.46 and 1.29 log for CTC1011, CTC1034 and Scott A, respectively. In products with 2.8% of added lactate, the difference in *L. monocytogenes* inactivation compared with the product without lactate was enhanced, indicating a piezo-protection effect due to the organic acid. For a holding time of 10 min, the difference in inactivation reached values of 2.51, 1.75 and 2.35 log for CTC1011, CTC1034 and Scott A, respectively, being relevant from a microbiological point of view (> 0.5 log) for holding times higher than 5 min. Diacetate had the opposite effect on *L. monocytogenes* inactivation compared to lactate as sensitized *L. monocytogenes* cells in front of the deleterious effects of HPP. For all studied strains, an enhanced HPP inactivation was observed as shown by the down left shift of the inactivation curves (Figure 1 j, k, l; Table 2) in comparison to the one obtained for control cooked ham. Time for the first log reduction (δ) was reduced by 13, 31 and 20 % in strains CTC1011, CTC1034 and Scott A, respectively, compared with the δ found in cooked ham without organic acids.

Interestingly, when organic acids salts (lactate and diacetate) were combined, the effects described above for each organic acid added alone were almost neutralized (Figure 1 m, n, o;, Table 2) and the *L. monocytogenes* inactivation curve was not statistically different (p-value>0.05) from that obtained in control cooked ham, indicating that for each *L. monocytogenes* strain, a common inactivation model could be used for control and 1.4 % lactate plus 0.1 % diacetate batches.

The mechanism by which the bacterial inactivation due to HPP is affected when salts of organic acids are added in the culture medium or food product has been scarcely studied. In the present study, while lactate was found to protect *L. monocytogenes* from HPP-inactivation, diacetate enhanced the lethal effect of pressure, indicating that probably *L. monocytogenes* used different mechanisms to respond to lactate and diacetate stresses. In the particular case of lactate, it has been hypothesized that the piezo-protection is related with the a_w decrease as a consequence of the addition of lactate in the product formulation (Shelef, 1994), but in the present study, the addition of organic acid salts did not change the physicochemical parameters of cooked ham compared to the control batch (Section 2.1), and thus, this seems unlikely to be the reason for the observed piezo-protection exerted by lactate on *L. monocytogenes*. Stasiewicz et al. (2011) reported that genes encoding membrane systems involved in ion transport and permeability were altered during adaptation of *L. monocytogenes* to growth on potassium lactate and diacetate. However, in the present study, *L. monocytogenes* was not grown in the presence of organic acids before the HPP, but was short-term exposed to the organic acid salts of the ham formulation from the moment of inoculation until pressurization of the samples (*ca*. 30 min). Therefore, molecular mechanisms behind a long-term adaptation and a short-term exposure to organic acids may not be the same. On the other hand, transcriptional activation of the general stress and oxidative stress responses have also been reported to be mechanisms used for bacteria for adaptation to organic acids' stress (Suo, Gao, Baranzoni, Xie, & Liu, 2018) and to HPP (Jofre, Garriga, & Aymerich, 2007; Bowman, Bittencourt, & Ross, 2008). Maybe, these genes and proteins could also play a role in *L. monocytogenes* inactivation.

Although the mechanisms involved in the piezo-protective effect of lactate have not been studied, cross-resistance effects between different stresses applied simultaneously have been described by some authors. Higher resistance of *L. monocytogenes* Scott A strain to HPP in semi-skimmed milk than in buffer was reported by Karatzas & Bennik (2002), showing a cross-resistance effect of HPP with the food matrix components, though no specific piezo-protective compound was identified. The HPP-induction of genes encoding cold-shock proteins suggested a cross-resistance with other stresses such as heat stress (Bowman et al., 2008). In another study carried out on brain heart infusion (BHI) broth, pre-exposure of *L. monocytogenes* H7858 strain to organic acid salts (i.e. lactate) induced a cross-protection (i.e. reducing

the sensitivity) against other food antimicrobials (nisin and ε–polylysine), being associated with the VirR-mediated genes (Kang, Wiedmann, Boor, & Bergholz, 2015). Additional genomic and transcriptomic studies would be necessary to understand the molecular basis of the piezo-protective effect of lactate on *L. monocytogenes* HPP inactivation.

3.3 Quantification of the dose-dependent piezo-protection of lactate

Despite the available data (Table 1) indicate that lactate protects *L. monocytogenes* from HPP inactivation, to the authors' knowledge, the quantification of this piezo-protective effect has not been performed before. This issue was addressed in the present study through a secondary and global modelling approach. Results are reported in Figure 2, which shows the effect of lactate on Weibull inactivation kinetic parameters, δ and p.

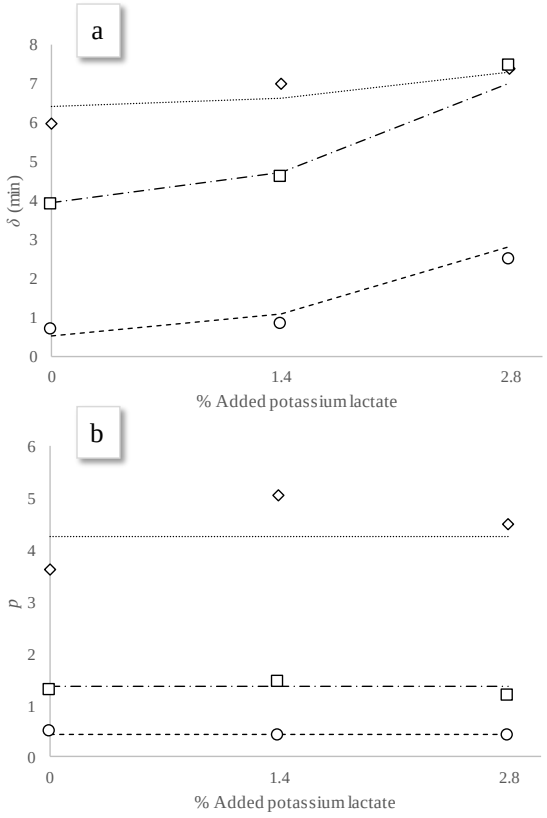

Figure 2. Effect of lactate on the holding time for the first log reduction (δ, plot a) and the shape parameter of the inactivation curve (p, plot b) of each *L. monocytogenes* strain. Diamonds, square and circle symbols represent the kinetic parameters of strains CTC1011, CTC1034 and Scott A, respectively. Secondary model fits for δ are shown in dashed lines.

The value of δ increased with increasing lactate concentration in a strain-dependent manner, especially for CTC1034 and Scott A. Despite of the transformations (square root, inverse, ln and log) assessed, none of the δ transformations contributed to develop a polynomial model with a better goodness of fit (data not shown). Therefore, non-transformed δ values obtained in the primary modelling were used to develop the models, being the independent term and the quadratic term of the polynomials statistically significant. F-test confirmed the need of three models to quantify the impact of lactate on δ for the three *L. monocytogenes* strains.

The p parameter values, which determine the shape of the inactivation curve, were not significantly dependent on lactate concentration as shown in Figure 2. A lack of fit was obtained by fitting polynomials to p values and thus, a fixed value of p for each *L. monocytogenes* strain could be used to describe the shape of the inactivation curve in all the concentrations of lactate.

Global fitting of the global model (Eq. 2) to 75 inactivation data points (log N/N_0) for each *L. monocytogenes* strain resulted in readjusted values of the terms describing the inactivation parameters δ and p (Table 3)

describing satisfactorily the lactate dose-dependent relationship magnitude of the inactivation but also the piezo-resistance characteristics of each strain.

Table 3. Parameter estimates for the global regression model for the inactivation of *L. monocytogenes* in cooked ham pressurized at 400 MPa formulated with lactate, obtained for three *L. monocytogenes* strains (CTC1011, CTC1034 and Scott A).

Strain	Kinetic parameters				Goodness of fit		
	$(\log N/N_0)_i$	δ(min)		p	RSS	RMSE	R^2_{adj}
		a	*b*				
CTC1011	-0.05	6.42	0.11	4.25	24.667	0.577	0.920
CTC1034	-0.14	3.94	0.39	1.35	39.737	0.733	0.659
Scott A	-0.18	0.53	0.29	0.43	38.715	0.728	0.622

[a] *$(\log N/N_0)i$* is a fixed value representing the average value of the initial bacterial inactivation of 3 replicates at holding time $t = 0$ (HPP treatment consisting in pressure come-up followed by an immediate pressure release), δ: pressure holding time to cause the first log reduction; p: shape of the inactivation curve (dimensionless).
[b] RSS: residual sum of squares; RMSE: root mean squared error; R^2_{adj}: adjusted coefficient of determination.

The developed models are particularly useful to assess HPP efficacy and find the processing parameters needed to achieve a specific *L. monocytogenes* inactivation and ultimately to comply with the safety standards requested by, for instance, international organizations regarding *L. monocytogenes* in ready-to-eat foods. Under the zero tolerance of the USA administration or the British Retail Council (BRC) Certification, a HPP-based post-lethality treatment has to be validated to achieve at least 1 log reduction of the pathogen level. When validated for a 2 log reduction an "increased level of control" is recognized by these institutions (FSIS, 2015; BRC Global Standards, 2018).

Using the developed model, the minimum treatment time at 400 MPa necessary to achieve a 2 log reduction and thus, to increase the expected level of control towards *L. monocytogenes*, can be estimated depending on the product formulation (Table 4). Interestingly, for CTC1011 strain, a 1.2 min increase in the holding time increases the inactivation of the pathogen from 1 log to 2 log in all lactate concentrations. Conversely, lower holding times are required to reduce 1 log of CTC1034 and Scott A strains in cooked ham formulated without lactate (2.72 and 1.77 min, respectively) but more than 1 extra minute is needed to achieve the 2 log reduction (2.7 and 1.8 additional min, respectively), showing higher resistance to higher holding times for these strains (Table 4).

Table 4. High pressure holding times necessary to cause the 1st and 2nd log reduction of *L. monocytogenes* strains (CTC1011, CTC1034 and Scott A) in cooked ham HP treated at 400 MPa and at different lactate concentrations predicted from global models of Table 3.

	Time for 1st log reduction (min)			Time for 2nd log reduction (min)		
Lactate (%)	CTC1011	CTC1034	Scott A	CTC1011	CTC1034	Scott A
0.0	6.34	3.52	0.33	**7.51**[a]	6.24	2.10
0.5	6.37	3.61	0.38	**7.54**	6.40	2.39
1.0	6.44	3.88	0.52	**7.63**	6.87	3.26
1.5	6.58	4.32	0.75	**7.79**	7.65	4.72
2.0	6.76	4.93	1.07	8.01	**8.74**	6.75
2.5	7.00	5.73	1.48	8.29	**10.15**	9.37
2.8	7.17	6.29	1.77	8.49	11.14	**11.22**

[a]: numbers in bold highlight the longest holding time of HPP to achieve 2 log reduction for each lactate concentration. It facilitates the identification of the most resistant strain depending on the lactate added in the cooked ham.

Moreover, differences in time for the first and second log reduction increment with increasing lactate concentrations, being necessary more than 11 min of pressurization (holding time) to achieve a 2 log reduction of the levels of the strains CTC1034 and Scott A in products with 2.80% of lactate.

4 Conclusions

The quantitative modelling approach allowed the characterisation of the lethal effect of HPP on *L. monocytogenes*, showing strain-dependent inactivation curves including convex (i.e. with a shoulder of survival cells during the first minutes of the treatment), linear (i.e. constant inactivation along treatment time) and concave (i.e. indicating the occurrence of a tail of resistant cells), which can be probably related with different molecular mechanisms of response to HPP depending on the strain. Interestingly, the presence of lactate exerted a notable and dose-dependent piezo-protective effect on *L. monocytogenes* in cooked ham but did not modify the strain-specific shape of the inactivation curve. The results showed that for the selection of the pathogen strain to be used for validating HPP, both the HHP duration and lactate concentration (as piezo-protective factor) are of paramount importance. As a result of this work, a versatile *L. monocytogenes* pool consisting of strains with different inactivation characteristics was obtained, which can be used in HPP validation studies for cooked meat products formulated either without or with organic acids.

This study emphasizes that the design, validation and implementation of high-pressure processing requires a tailor-made approach, considering the specific product formulation and the selection of the most appropriate strain/s.

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7 Supplementary data

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Article 5

Unravelling the molecular mechanisms underlying the protective effect of lactate on the high-pressure resistance of *Listeria monocytogenes*

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Unravelling the Molecular Mechanisms Underlying the Protective Effect of Lactate on the High-Pressure Resistance of *Listeria monocytogenes*

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Abstract: Formulations with lactate as an antimicrobial and high-pressure processing (HPP) as a lethal treatment are combined strategies used to control *L. monocytogenes* in cooked meat products. Previous studies have shown that when HPP is applied in products with lactate, the inactivation of *L. monocytogenes* is lower than that without lactate. The purpose of the present work was to identify the molecular mechanisms underlying the piezo-protection effect of lactate. Two *L. monocytogenes* strains (CTC1034 and EGDe) were independently inoculated in a cooked ham model medium without and with 2.8% potassium lactate. Samples were pressurized at 400 MPa for 10 min at 10 °C. Samples were subjected to RNA extraction, and a shotgun transcriptome sequencing was performed. The short exposure of *L. monocytogenes* cells to lactate through its inoculation in a cooked ham model with lactate 1h before HPP promoted a shift in the pathogen's central metabolism, favoring the metabolism of propanediol and ethanolamine together with the synthesis of the B12 cofactor. Moreover, the results suggest an activated methyl cycle that would promote modifications in membrane properties resulting in an enhanced resistance of the pathogen to HPP. This study provides insights on the mechanisms developed by *L. monocytogenes* in response to lactate and/or HPP and sheds light on the understanding of the piezo-protective effect of lactate.

Keywords: *Listeria monocytogenes*; pressurization; HPP; organic acids; piezo-resistance

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1. Introduction

Listeria monocytogenes is a facultative anaerobic Gram-positive pathogen that can cause listeriosis, with several outbreaks being associated with ready-to-eat (RTE) products. The risk assessments developed so far indicate that within the RTE meat products, cooked meat products have to be considered of high risk due to the exposure to recontamination with *L. monocytogenes* during the preparation of convenient formats (i.e., sliced/diced and packaged) and due to the potential of *L. monocytogenes* to grow during the refrigerated storage thanks to its psychrotrophic nature [1].

Differences in food safety microbiological criteria regarding *L. monocytogenes* are found between countries, setting from a maximum of 100 CFU/g of *L. monocytogenes* during the shelf-life of the product in EU [2] to the zero-tolerance policy (not detected in 25 g) in USA [3]. In this regard, control measures can be implemented by food manufacturers to comply with the legislation by minimizing the prevalence of the pathogen as well as by limiting its growth in contaminated products.

Among all the available control strategies, high pressure processing (HPP) is an emergent non-thermal technology widely applied in the meat industry. HPP is often used

as a post-lethality treatment (PLT) with the aim of reducing microbial loads in foods that have been exposed to microbial recontamination before their commercialization, i.e., during slicing and packaging operations [4]. Another control measure frequently used by the meat industry to prevent the growth of *L. monocytogenes* is the use of antimicrobial agents (AMA), especially organic acids and/or their salts [5]. It is known that organic acids in a medium exist in equilibrium between the undissociated and dissociated state, the former being able to cross the cell membrane entering into the cell, where it dissociates liberating anions [6]. The presence of an increased amount of lactate anions inside the cell increases the osmotic pressure and affects the functioning of the cell metabolism, thus resulting in an impaired bacterial growth.

In some cases, the combined application of HPP and organic acid salts is chosen by the food industry in order to comply with the highest level of control of *L. monocytogenes* requested in Alternative 1 (combination of a PLT and an AMA) under the requirements of zero tolerance policy of USA [3]. According to the hurdle technology concept described by Leistner [7], the intelligent combination of hurdles (as sub-lethal stresses) leads to an increased effectiveness in controlling *L. monocytogenes* survival/growth. However, cross-protection of a sublethal stress against subsequent treatments can also occur, damaging cells without killing them [8]. Few studies have been conducted dealing with the effect of combination of strategies (i.e., HPP and organic acids) on *L. monocytogenes* in meat products [4,9]. Interestingly, Serra-Castelló et al. [10] showed that the HPP inactivation of three *L. monocytogenes* strains (CTC1034, CTC1011 and Scott A) in cooked ham formulated with potassium lactate was lower than in cooked ham without this antimicrobial. This piezo-protective effect was quantified showing it was strain and lactate dose-dependent. Additionally, in cooked meat products, *L. monocytogenes* surviving HPP was found to grow at higher rate compared to non-pressurized *L. monocytogenes* during the storage of the products [11], such piezo-stimulation effect was enhanced in products formulated with lactate [11].

The present study aimed to investigate by means of transcriptomics the molecular mechanisms underlying the piezo-protective effect exerted by lactate on *L. monocytogenes* HPP inactivation in a cooked ham model medium.

2. Material and Methods

2.1. Cooked Ham Model Medium Formulation and Characterization

Cooked ham model medium (CHMM) was prepared with Brain Heart Infusion (BHI) broth (Beckson Dickinson, Sparks, MD, USA) and the addition of the following ingredients (g/L) usually used in the manufacture of cooked ham from pork meat: sodium chloride, 15.7; dextrose, 5.77; sodium ascorbate, 0.6; and sodium nitrite, 0.1. The medium was sterilized at 121 °C for 20 min. In order to have samples without organic acids (control) and with lactate, two lots of CHMM were prepared: without and with 2.8% (*v/v*) potassium lactate (using HiPure P Plus, Corbion©, Montmeló, Spain, known to have 76–80% *w/w* of potassium lactate).

2.2. L. monocytogenes Strains and Pre-Culture Conditions

Strains of *L. monocytogenes* used in the present study included two different serotypes with relevance from the clinical and from the food and food processing environment perspective [12]. The meat isolate CTC1034 (serotype 4b) from the IRTA Food Safety Program's collection and previously used in studies dealing with the application of HPP in meat products [10,11,13,14] and the *L. monocytogenes* strain EGDe (serotype 1/2a) as a reference strain. For this study, three biological replicates of each strain were prepared from −80 °C stock cultures.

L. monocytogenes strains CTC1034 and EGDe were refreshed into 8 mL of BHI broth for 7 h at 37 °C. Afterwards, 1% (*v/v*) were consecutively subcultured in 200 mL of fresh BHI at 37 °C for 14 and 24 h, respectively, in order to standardize the strains at the early stationary

phase. After incubation, cultures were preserved frozen at −80 °C supplemented with 20% of glycerol until used [15].

2.3. Preparation of the Samples and HPP

For each biological replicate, cultures of *L. monocytogenes* strains CTC1034 and EGDe were thawed at ambient temperature and centrifuged at 8240× *g* for 7 min at 12 °C. Supernatants were discarded and cell pellets were resuspended in the same volume of CHMM without or with 2.8% of lactate. Cultures were distributed in 4 × 10 cm PA/PE pouches (oxygen permeability of 50 $cm^3/m^2/24$ h and a low water vapor permeability of 2.8 $g/m^2/24$ h; Sistemvac, Estudi Graf S.A., Girona, Spain), which were closed by thermosealing. Cultures were kept for 1 h at 10 °C to allow the adaptation of *L. monocytogenes* cells in CHMM medium without and with 2.8% of lactate. Half of the samples were subsequently pressurized at 400 MPa for 10 min using an industrial HPP equipment (Wave 6000; Hiperbaric, Burgos, Spain). The come-up time was 2.50 min and the pressure release time was almost immediate (<2 s). The pressurization fluid was water and the initial temperature was set at 10 °C. After pressurization, samples were kept for 30 min at 10 °C before *L. monocytogenes* enumeration and RNA extraction. Non-pressurized samples were kept at 10 °C until analysis together with the HPP samples.

2.4. L. monocytogenes Enumeration and Data Analysis

For each treatment and biological replicate, *L. monocytogenes* concentration was determined by plate colony count method from the appropriate tenfold serial dilution prepared in 0.1% Bacto Peptone (Difco Laboratories, Detroit, MI, USA) with 0.85% NaCl. Samples were spread on CHROMagar™ Listeria (CHROMagar, Paris, France) and incubated at 37 °C for 48 h according to the manufacturer instructions. Chromogenic media for *L. monocytogenes* are known to be able to recover high pressure injured *L. monocytogenes* [16,17]. In any case, plates were further checked after additional 24–48 h to make sure that sub-lethally injured cells had time to recover and form colonies and, thus, minimize the overestimation of the lethal effect of HPP [10,11]. *L. monocytogenes* counts were Log transformed, and the inactivation value in terms of Log reduction was calculated by subtracting from the counts found in non-pressurized cultures (Log N_0) those of the pressurized cultures (Log N), i.e., $LogN_0 - LogN = Log\ N_0/N$, both in the control and 2.8%-lactate lots.

2.5. Nucleic Acid Extraction and Sequencing

DNA of the samples prepared according to Section 2.3 was extracted from *L. monocytogenes* strain CTC1034 by using 1 mL of an overnight culture of BHI centrifuged at 14,000× *g* for 10 min. The pellet was then used for DNA extraction according to the protocol described in Cocolin et al. [18]. DNA was quantified using the QUBIT DS-HS kit (Thermo Fisher Scientific, Milan, Italy) and it was standardized at 50 ng/µL. Whole genome sequencing (WGS) was performed using NEBNext® library prep Kit according to the manufacturers' instructions in paired-end (2 × 150 bp) on a NextSeq 550 Illumina system by the Novagene Company (Cambridge, United Kingdom).

For the transcriptomic analysis, *L. monocytogenes* cultures of CTC1034 and EGDe strains were centrifuged at 10,416× *g* for 5 min at 10 °C and pellets corresponding to 3.6 mL of culture were resuspended with 125 µL of RNAlater solution (Invitrogen, Thermo Fisher Scientific, Barcelona, Spain,) and kept at −80 °C. Total RNA was extracted from the pellets using the RNeasy PowerMicrobiome Kit (QIAGEN, Hilden, Germany) following the manufacturers' instructions, and residual DNA was removed with TURBO DNase (Invitrogen, Thermo Fisher Scientific, Milan, Italy) according to the manufacturers' instructions. RNA concentrations were quantified by using a Nanodrop Instrument (Spectrophotometer ND-1000, Thermo Fisher Scientific, Milan, Italy). The RNA integrity was verified by agarose gel electrophoresis. The RNA sequencing library preparation and cDNA synthesis were performed using the NEBNext Ultra RNA Library Prep Kit according to the manufacturers' instructions at Genewiz Inc. (Leipzig, Germany). The transcriptome was studied for all the

samples from the experiment and sequencing was carried out on a NextSeq 550 Sequencer yielding 150 bp paired-end reads.

2.6. Bioinformatics and Data Analysis

WGS of *L. monocytogenes* strains CTC1034 led to 5,484,770 paired-end reads. Low-quality bases (Phred score < 20) were trimmed, and reads shorter than 60 bp were discarded using the SolexaQA++software v3.1.7.1 and PRINSEQ v0.20.4, respectively [19,20]. Reads were assembled using SPAdes v3.14.1 [21]; genes were annotated with Prokka v 1.14.5 [22] and used to build the reference database. A draft genome of *L. monocytogenes* EGDe (NC_003210.1) was downloaded from NCBI (BioProject: PRJNA61583), and genes were annotated with Prokka. The pangenome calculation and phylogenetic analysis of *L. monocytogenes* strains were obtained by Roary v. 3.11.2 [23].

In order to investigate the molecular background that could explain the observed differences in the inactivation between the two *L. monocytogenes* strains as well as the piezo-protective effect of lactate, a transcriptomic approach was implemented. Total RNA was extracted, sequenced, and compared between *L. monocytogenes* cultures shortly exposed to (i) CHMM (control without HPP), (ii) CHMM supplemented with lactate (without HPP), (iii) CHMM and subjected to HPP, and (iv) CHMM supplemented with lactate and subjected to HPP.

Raw reads were quality filtered by SolexaQA++ software and PRINSEQ (Phred score < 20, <60 bp). Reads were aligned against the respective build database by using Bowtie2 in end-to-end, sensitive mode according to the strain used. The number of reads mapped to each gene (*.sam* files) were then used for KEGG functional analysis using MEGAN6 software [24]. Data normalization and determination of differentially abundant KEGG genes, among the studied conditions (lactate and HPP, alone, or in combination) or strains, were conducted using the Bioconductor DESeq2 package [25] in the statistical environment R [26] with default parameters. The statistical significance (*p*-values) was adjusted for multiple testing using the Benjamini–Hochberg procedure, which assesses the false discovery rate (FDR) by using the DESeq2 package.

Gene set enrichment for pathway analysis was then performed on KEGG orthologs table imported in the *GAGE* Bioconductor package [27] to identify biological pathways overrepresented or underrepresented between sample without lactate and without HPP treatment against the other combination.

2.7. Availability of Data and Material

WGS and Metatranscriptomic raw sequence reads were deposited at the Sequence Read Archive of the National Center for Biotechnology Information (Bioproject accession number: PRJNA692371 and PRJNA692360, for *L. monocytogenes* CTC1034 and EGDe, respectively).

2.8. Fatty Acid Profile of L. monocytogenes

For the strain CTC1034 the fatty acid profile was analyzed to confirm potential changes in the membrane composition due to exposure to lactate and/or HPP. For this, samples of *L. monocytogenes* CTC1034 were centrifuged at 10,416× *g* for 6 min at 10 °C. Supernatant was discarded and pellets were resuspended in 1 mL of purified water. Cells were disrupted with 0.5 g of glass beads in a mixer mill (Mixer Mill MM200, Retsch, Llanera, Spain) for 5 min at 30 Hz, centrifuged and supernatant was discarded. Pellets were frozen at −20 °C for 2 h before being freeze dried (Lyomicron LM-181004, Coolvacuum, Granollers, Spain). Methyl esters of fatty acids (FAME) were obtained by methylation described by Castro-Gómez et al. [28], using tritridecanoine as an internal standard. FAME analysis was carried out on an Autosystem chromatograph (Perkin Elmer, Beaconsfield, UK) fitted with a VF-23ms, fused silica capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness, Varian, Middelburg, The Netherlands) and FID, according to Calvo et al. [29]. The statistical difference of the results among conditions was assessed through the MANOVA test.

3. Results and Discussion

3.1. Inactivation of L. monocytogenes by HPP

Inactivation of *L. monocytogenes* strains CTC1034 and EGDe submitted to HPP at 400 MPa for 10 min in the CHMM resembling the composition of a cooked ham, with or without potassium lactate, is shown in Figure 1. The results show that the application of HPP in a medium without lactate inactivated CTC1034 and EGDe strains by an average reduction of 1.17 ± 0.20 and 2.96 ± 0.43 Log units, respectively. Thus, the strain CTC1034 was significantly ($p < 0.05$) more resistant to HPP than EGDe. In the presence of lactate in the CHMM, HPP resulted in a lower inactivation of the strains, recording 0.44 ± 0.04 and 2.36 ± 0.22 Log reduction for CTC1034 and EGDe, respectively. In particular, for the CTC1034 strain, the lethal effect of HPP was lower ($p < 0.05$) in the presence of lactate, corroborating the piezo-protective effect of this antimicrobial on *L. monocytogenes* inactivation as previously shown for this and other strains inoculated in different types of meat products [9,10,30,31].

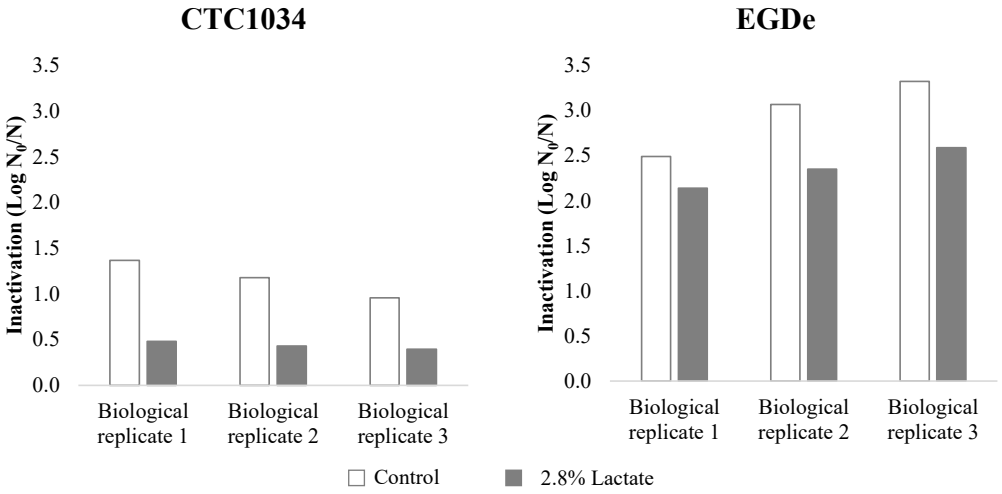

Figure 1. Inactivation (Log N_0/N) of each biological replicate of the *L. monocytogenes* CTC1034 and EGDe strains observed after HPP (400 MPa for 10 min) in cooked ham model medium without (control) and with 2.8% (*v/v*) potassium lactate.

3.2. Analysis of RNA-Seq Results. KEGG Annotation Classification and Pathway Enrichment Analysis of the DEGs

3.2.1. Comparison of *L. monocytogenes* CTC1034 and EGDe Genomes

WGS sequencing of *L. monocytogenes* CTC1034 showed a total of 19 contigs that provide a total genome length of 2,943,406 bp with an average GC content of 38.05%. Sequencing revealed the presence of 2958 CDS, 1 tmRNA and 61 tRNA encoding genes.

The comparison of *L. monocytogenes* genomes of CTC1034 and EGDe strains showed the presence of 2967 core genes including 394 genes encoding hypothetical proteins. Only 77 genes were absent or present in one *L. monocytogenes* strain compared to the other, 35 genes being found in CTC1034 but not in EGDe and 42 being found in EGDe but not in CTC1034. Most of the 35 genes found in CTC1034, but not in EGDe, were related to transcription factors, while the major fraction of genes found in EGDe were involved in protein export and transcription factors. As transcription factors regulate gene expression, a greater abundance in the CTC1034 could be related to the major resistance to HPP stress this strain has shown [32].

3.2.2. Whole Transcriptome Analysis

For the transcriptomic analysis involving both *L. monocytogenes* strains, a total of 152.43 Gbp of clean reads were obtained. For each sample, approximately 6.62 Gbp of reads

were found (Supplementary Table S1). The KEGG analysis assigned 864 genes to 24 KEGG pathways.

Results from the statistical analysis of the KEGG genes obtained with the transcriptomic analysis revealed that the number of differentially expressed genes (DEGs) found in the pairwise comparisons between all the condition combinations studied (effect of lactate, effect of HPP and effect of both factors) was strain-dependent (Figure 2; Supplementary Tables S2–S10).

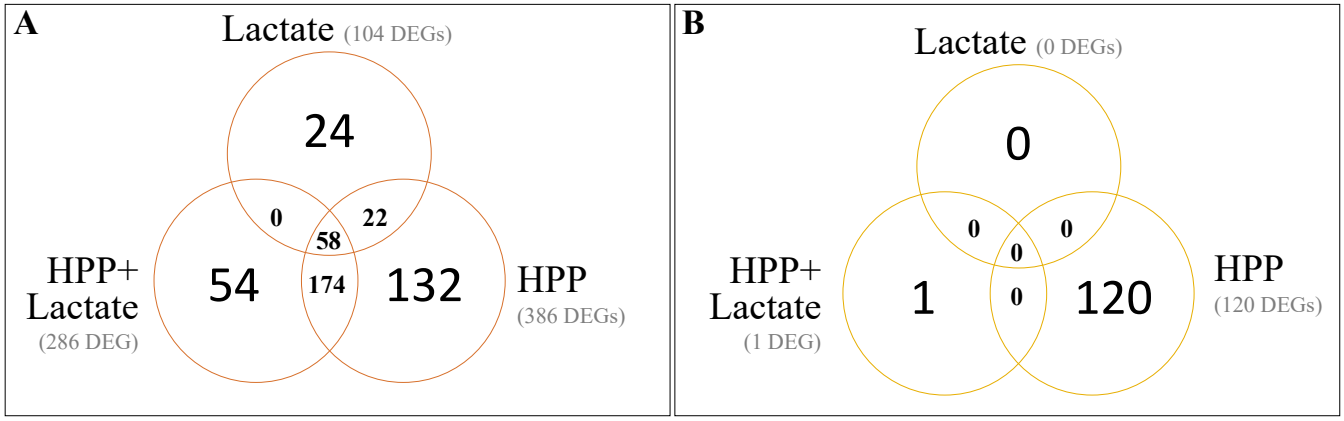

Figure 2. Venn diagrams of differentially expressed genes (DEGs) of *L. monocytogenes* strains CTC1034 (**A**) and EGDe (**B**) due to the exposure of cells to lactate, the application of the HPP (400 MPa for 10 min) and the application of both stresses compared to control conditions (exposed to CHMM without lactate).

In this framework, the stress induced by the exposure of *L. monocytogenes* cultures to CHMM with lactate compared to those exposed to CHMM without the antimicrobial resulted in a different response depending on *L. monocytogenes* strain. While the presence of lactate in the CHMM resulted in 104 DEGs in CTC1034, no DEGs were found in EGDe (Figure 2; Supplementary Table S3). A similar pattern was obtained when analyzing the effect of the application of both stresses, lactate and HPP, on *L. monocytogenes* compared to control conditions, resulting in 286 DEGs for the CTC1034 and only 1 DEGs for the EGDe strain (Figure 2; Supplementary Tables S6 and S10). Therefore, these results suggest that the response to stress is highly dependent on the particularities of the *L. monocytogenes* strain. In the study of the transcriptional response of two *L. monocytogenes* strains due to exposure to organic acids (lactate and diacetate) reported by Stasiewicz et al. [33], large differences on the number of transcribed genes were found and only a minor fraction of the differentially transcribed genes were shared between the two strains.

Additionally, it was interesting to observe that DEGs found for EDGe in the pairwise comparison of pressurized samples with and without the presence of lactate (Supplementary Table S8) were the same or involved in the same metabolic pathways as those DEGs found in non-pressurized cultures of CTC1034 in response to lactate stress (Supplementary Table S3). The different pairwise comparisons between the stressing conditions involving lactate also support this hypothesis (Supplementary Tables S4, S5, S8 and S9). These results would lead to the hypothesis that both *L. monocytogenes* strains employ similar molecular mechanisms in response to the lactate stress, although they seem to be activated in a different magnitude and/or time frame.

On the other hand, the application of the HPP resulted in 386 and 120 DEGs for the CTC1034 and EGDe strains, respectively, when compared to control conditions, i.e., *L. monocytogenes* cultures exposed to CHMM without lactate (Figure 2; Supplementary Tables S2 and S7).

The pathway enrichment analysis (performed by GAGE) of the KEGG genes of CTC1034 strains showed an enrichment of several pathways in CHMM subjected to HPP (with and without lactate) compared with the control CHMM (without HPP nor lactate), including Flagellar assembly (ko02040), Fructose and mannose metabolism (ko00051), Phosphotransferase system (ko02060), Biosynthesis of amino acids (ko01230) and Pheny-

lalanine, and tyrosine and tryptophan biosynthesis (ko00400). Moreover, an enrichment of the flagellar assembly (ko02040) and a reduction in glycolysis/gluconeogenesis (ko00010) in CHMM supplemented with lactate without HPP was observed when compared with CHMM. Regarding EGDe, an enrichment in cysteine and methionine metabolism (ko00270), peptidoglycan biosynthesis (ko00550), fatty acid metabolism (ko01212), biosynthesis of amino acids (ko01230) and citrate cycle (ko00020), and a downregulation of the flagellar assembly (ko02040) and phosphotransferase system (PTS) (ko02060) were observed in CHMM subjected to HPP if compared with non-pressurized CHMM (data not shown).

3.2.3. Effect of Lactate Exposure on *L. monocytogenes*

Some studies support that in order to counteract the intracellular osmotic pressure caused by an increased amount of lactate, bacteria (i) reduce intracellular pools of anions and (ii) shift the flux in the central carbon metabolism [34]. The results from the present transcriptomic analysis reveal that *L. monocytogenes* could use both strategies to overcome the stress suffered by its exposure to lactate. Regarding the possible effect of lactate on the central carbon metabolism of the pathogen, the results of the present study show that genes involved in the pentose phosphate pathway coupled with oxidative reactions to produce reducing equivalents (*rpiB*, *tktA*, *tktB*, *G6PD*) were upregulated. Additionally, a downshift was observed in the conversion of pyruvate to acetyl-CoA and ethanol, as indicated by the downregulation of genes such as *pdhC*, *plfD*, and *adhE*. In line with the output of the pathway enrichment analysis described above, these transcriptomic results suggest that in presence of lactate, *L. monocytogenes* redistributed its metabolic carbon flux from the glycolytic pathway to oxidative reactions producing reducing equivalents (Figure 3).

Genes of other metabolic pathways that are source of reducing equivalents were also upregulated (Figure 3). In this framework, genes involved in the synthesis of cobalamin and corrinoid cofactors and B12 cofactor (adenosylcobalamin) (*CbiK-CbiX, CbiL, CobI, CbiH, CobJ, CbiF, CobM, CbiD, CbiT, CbiC, CobH, CbiA, CobB, CbiB, CobC, CobD, CobU, CobS, CobV,* and *EutT*), which consist of reductive reactions, were also found to be upregulated (Supplementary Tables S3 and S8). In addition, the higher expression of genes related to the cobalamin and corrinoid pathways is coordinated with the upregulation of the genes involved in the 1,2 propanediol (*PduC*, *PduD*, *PduE*, *PduP*, *PduQ*, *PduL PduW*) and ethanolamine metabolism (*EutH*, *EutA*, *EutB*, *EutC*, *EutQ, EutN, EutJ, EutT, EutL*) found in the presence of lactate (Supplementary Tables S3 and S8), since both pathways are regulated by the cofactor B12 riboswitch in *L. monocytogenes*, the synthesis of the cofactor B12 being required for the metabolism of these pathways [35]. Such coordination is biologically relevant since the B12 cofactor is required in the catabolic pathways of ethanolamine and propanediol degradation. Moreover, genes involved in the catabolism of rhamnose (*rhaA*, *rhaB*, *dhal* and *glpK*) were upregulated, suggesting that it can be used as a carbon source for the 1,2 propanediol pathway [36]. The use of 1,2 propanediol and ethanolamine as a carbon source has been reported to provide a competitive advantage to *L. monocytogenes* under diverse conditions such as when growing in vacuum-packaged smoked salmon [37] or when co-cultured with other bacteria [38]. In the present study the role of 1,2 propanediol and ethanolamine metabolism in the piezo-protective effect of lactate on *L. monocytogenes* could not be directly elucidated, but they are important metabolites that provide a fitness advantage to *L. monocytogenes* [39].

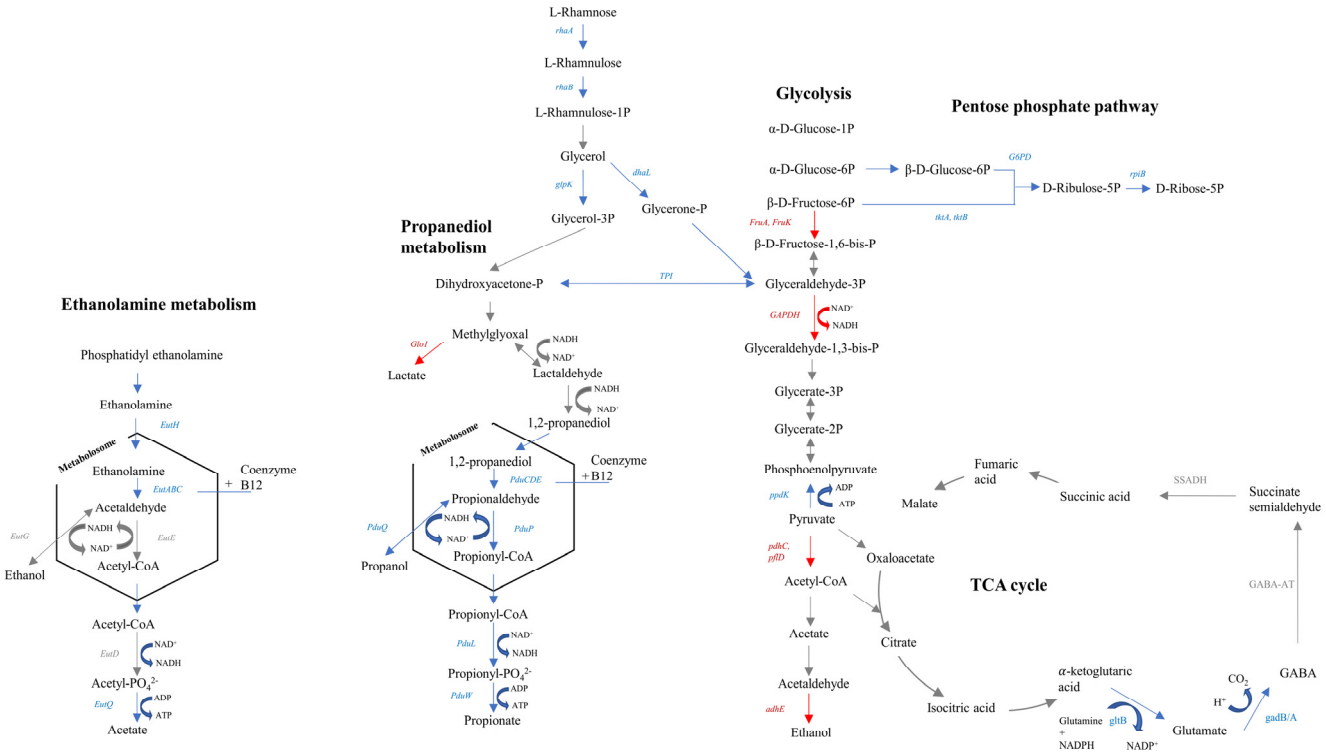

Figure 3. Predicted carbon flux in *L. monocytogenes* CTC1034 and EGDe when exposed to lactate. Blue, red, and grey arrows and text indicate genes that were upregulated, downregulated, or were not differentially expressed, respectively. Genes and proteins: *EutH*, ethanolamine transporter; *EutA*, ethanolamine transporter protein EutA; *EutB*, ethanolamine ammonia-lyase large subunit; *EutC*, ethanolamine ammonia-lyase small subunit; *EutG*, alcohol dehydrogenase; *EutE*, aldehyde dehydrogenase; *EutD*, phosphotransacetylase; *EutQ*, ethanolamine utilization protein EutQ; *Glo1*, lactoylglutathione lyase; *PduC*, propanediol dehydratase large subunit; *PduD*, propanediol dehydratase medium subunit; *PduE*, propanediol dehydratase small subunit; *PduP*, propionaldehyde dehydrogenase; *PduQ*, 1-propanol dehydrogenase; *PduL*, phosphate propanoyltransferase; *PduW*, propionate kinase; *TPI*, triosephosphate isomerase; *FruA*, fructose PTS system EIIBC; *FruK*, 1-phosphofructokinase; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *ppdK*, pyruvate orthophosphate dikinase; *pdhC*, pyruvate dehydrogenase E2 component; *pflD*, formate C-acetyltransferase; *adhE*, acetaldehyde dehydrogenase/alcohol dehydrogenase; *tktA*, *tktB*, transkelotase; *G6PD*, glucose-6-phosphate 1-dehydrogenase; *RpiB*, ribose 5-phosphate isomerase B; *DhaL*, phosphoenolpyruvate-glycerone phosphotransferase subunit DhaL; *GlpK*, glycerol kinase; *RhaB*, rhamnulokinase; *RhaA*, L-rhamnose isomerase; *gltB*, glutamate synthase; *gadB/A*, glutamate descarboxylase; *GABA-AT*, GABA aminotransferase; *SSADH*, succinate semialdehyde dehydrogenase

Together with lactate anions, protons are also accumulated inside the cell, with the consequent disruption of bacterial transmembrane potential. In this framework, one of the strategies frequently used by bacteria to restore intracellular pH homeostasis and/or maintain transmembrane potential is the metabolism of glutamate [40,41]. The intracellular decarboxylation of glutamate by a glutamate decarboxylase enzyme to form aminobutyric acid (GABA) results in the consumption of one proton, contributing to restore the intracellular pH [42]. The upregulation of genes involved in the metabolism of glutamate (*gadAB*, *gltBD)* pointed out that *L. monocytogenes* could use this strategy to restore intracellular pH homeostasis disturbed when exposed to lactate (Figure 3; Supplementary Tables S3 and S8).

The enrichment of flagellar assembly pathways and in detail of flagellar genes (*FlhA*, *FlhF*, *FliC*, *FliE*, *FliF*, *FliG*, *FliH*, *FliI*, *FliR*, *FliP*, *FlgB*, *FlgC*, *FlgD*, *FlgE*, *FlgG*, *FlgK*, and *FlgL*) found in the presence of lactate (Supplementary Tables S3 and S8) could indicate that the electrochemical potential of protons across the cytoplasmic membrane could also contribute to fuel the flagellar motor of the pathogen [43] and/or that the unfavorable environment faced by *L. monocytogenes* would promote the pathogen to elicit the chemotactic response and to move to a more favorable environment [44].

The activation of all the strategies to counteract the osmotic pressure and membrane potential changes due to lactate would result in less efficient pathways for ATP production and in a higher energy expenditure, leading to the limitation of growth in the presence of lactate [45–47]. A decrease of metabolic energy generation due to the increase in external lactate concentration was described in *Streptococcus cremoris* [48].

In addition to the up/downregulation of molecular mechanisms involved in restoring osmotic pressure and membrane potential, it is worth to highlight that in the presence of lactate, *L. monocytogenes* specifically upregulated genes involved in the methionine synthesis (Figure 4), in particular a higher expression of the methyltransferases *mmuM* in CTC1034 (Supplementary Table S3) and *MetE* in pressurized EGDe (Supplementary Table S8) was found. Both enzymes are responsible for converting homocysteine to methionine, thus suggesting that in the presence of lactate *L. monocytogenes* promoted the oxidation of homocysteine to methionine, avoiding the accumulation of the toxic metabolite homocysteine and increasing the amount of intracellular methionine. In accordance with this, genes associated with the sulfur metabolism (*metC*, *metX*, *cysE* or *cysO*) involved in the methionine synthesis were also found to be upregulated by the exposure of *L. monocytogenes* to lactate (Figure 4; Supplementary Tables S3 and S8). In previous studies dealing with the transcriptome analysis of *L. monocytogenes* cells exposed to lactate, the upregulation of the methionine biosynthesis was not reported [33,49]. However, in those experiments *L. monocytogenes* was exposed to lactate for a much longer time, i.e., 8 h at 7 °C and 48 h at 15 °C, than the exposure time used in the present study (<2 h at 10 °C). It can be hypothesized that the upregulation of the methionine synthesis would only occur in the early exposure of the pathogen to lactate as a first step of the overall mechanism to overcome the stress suffered by the presence of lactate. In addition to the time-related factor, other potential reasons leading to different results include the pathogen strains, the concentration and the type of salt (sodium vs. potassium), and the incubation temperature or the matrix composition (culture medium) used for the experiment.

Among all the multiple factors that can determine the expression of genes involved in the methionine synthesis, the observed upregulation of this metabolic pathway by *L. monocytogenes* in the presence of lactate could be relevant in relation to the piezo-resistance mechanisms since another organic acid such as acetate has been shown to specifically inhibit the synthesis of methionine in *Escherichia coli*, favoring the accumulation of the toxic compound homocysteine and consequently limiting or even inhibiting the growth of the pathogen [50]. Moreover, Roe et al. [50] reported that the addition of methionine in the medium containing acetate restores *E. coli* growth to 80% of that observed in medium without acetate, indicating that the inhibition of the methionine biosynthesis is one of the main factors responsible for the growth depletion of *E. coli* cultured in the presence of acetate. Supporting these results, Pinhal et al. [51] reported that the uncoupling effect of acetate or the perturbation of the anion composition of the cell played only a limited role (20%) in the *E. coli* growth depletion, suggesting that other molecular mechanisms, such as the inhibition of the methionine synthesis, could have a more prominent role on the bacterial growth-inhibitory effect.

Methionine can be converted to S-adenosyl-L-methionine (SAM), which represents a methyl group donor for many fundamental cellular processes, such as cellular signaling and epigenetic regulations that promote cellular anabolism and proliferation in bacteria and yeasts [52,53]. Specifically, SAM is involved in the methylation of proteins, RNAs, biotin, polyamines, and lipids [53,54]. In the present study, the *metK* gene responsible for the conversion of methionine to SAM was found to be upregulated in the *L. monocytogenes* CTC1034 strain when it was exposed to lactate, suggesting a higher production of SAM. Moreover, an increased intracellular concentration of methionine was also reported to contribute to the antioxidant defense in bacteria [55], although its role in the piezo-protection remains unknown.

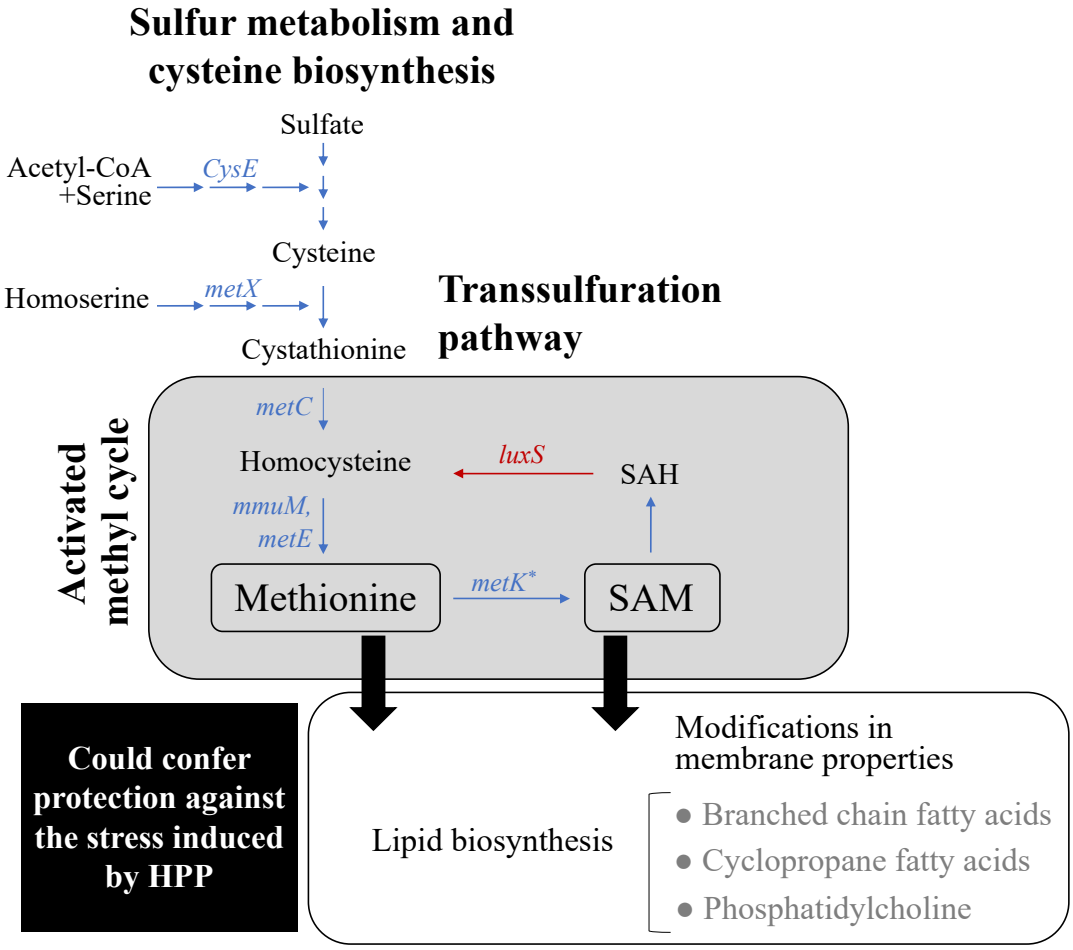

Figure 4. Predicted activation of the methyl cycle in *L. monocytogenes* CTC1034 and EGDe strains when exposed to lactate and its potential role on the piezo-protective effect exerted by lactate on *L. monocytogenes* stress induced by HPP. Blue and red arrows and text indicate genes that were upregulated and downregulated, respectively. Genes and proteins: *CysE*, serine O-acetyltransferase; *metX*, homoserine O-acetyltransferase; *metC*, cysteine-S-conjugate beta-lyase; *mmuM* and *metE*, homocysteine S-methyltransferases; *metK*, S-adenosylmethionine synthetase; *luxS*, S-ribosylhomocysteine lyase; *SAM*, S-adenosyl-methionine; *SAH*, S-adenosyl-homocysteine.

3.2.4. Effect of HPP on *L. monocytogenes*

The transcriptomic analysis revealed that both *L. monocytogenes* strains upregulated genes involved in DNA repair mechanisms such as *RadA*, *phrB*, *uvrB*, *adaB*, and lipid and peptidoglycan biosynthetic pathways (*glmS*, *murF*, *murG*, *murC*, or *fabH*), among others (Supplementary Tables S2 and S7), presumably as a consequence of the stress induced by the application of the HPP to *L. monocytogenes*. In case of flagella assemblage (*FlhA*, *FlhF*, *FliC*, *FliE*, *FliF*, *FliG*, *FliH*, *FliI*, *FliR*, *FliP*, *FlgB*, *FlgC*, *FlgD*, *FlgE*, *FlgG*, *FlgK* and *FlgL*) and chemotaxis (*MotA*, *CheA*, *CheR*, *CheY*, *FliG*, *FliM*, and *FliN/FliY*), an upregulation of genes involved in these pathways was found in CTC1034 (Supplementary Table S2), while a downregulation was observed in EGDe (Supplementary Table S7). These differences could be related to the particularities of each *L. monocytogenes* strain but also to the higher severity of the HPP injury in the EGDe strain compared to CTC1034, leading to a higher inactivation extent (Figure 1). An important parameter influencing motility of *L. monocytogenes* is temperature; *L. monocytogenes* cells are motile at temperatures below 30 °C but not at human body temperature (37 °C) [56]. Additionally, flagella, as cell surface appendices, are considered putative virulence factors. In the current study, the temperature for the experiments could partially explain the upregulation of the flagella genes in CTC1034. In addition to this, we may deduce that these genes would be downregulated when

L. monocytogenes is under stress (for example desiccation) [57]. It is therefore puzzling that HPP resulted in an upregulation in CTC1034, and at this point we cannot provide a biological explanation. Nevertheless, this observation is particularly relevant since it suggests that cells of *L. monocytogenes* surviving the HPP treatment would be prepared to colonize the human body [58]. On the other hand, HPP was found to downregulate genes involved in the septal ring (*ftsA*, *ftsW*, *ftsQ*, *mreB*). These results were in line with those reported by Bowman et al. [59] regarding the response of *L. monocytogenes* pressurized at 400–600 MPa for 5 min in tryptone soy yeast extract (TSYE) broth.

As a response to HPP, *L. monocytogenes* CTC1034 and EGDe upregulated genes involved in the methionine biosynthesis (*luxS*, *mmuM*, *msrB*), suggesting an enhanced methionine production/availability (Supplementary Tables S2 and S8), which also agrees with the enrichment gene analysis for EGDe (see Section 3.2.2). The upregulation of these genes pointed out that, as stated due to the exposure to lactate (Section 3.2.3), the application of HPP would result in a higher generation of SAM in *L. monocytogenes*, which could affect cellular processes throughout its role in the methyl cycle [60]. These results are in accordance with those reported by Bravim et al. [61], where it was found an upregulation of the sulfur metabolism genes involved in the activation of the methionine biosynthesis when *Saccharomyces cerevisiae* was submitted to an HPP of 50 MPa for 30 min.

Considering the metabolic pathways in which methionine and SAM are involved, methionine could increase *L. monocytogenes* resistance to HPP for its role as an endogenous antioxidant in cells [62] and for its involvement in lipid biosynthesis [63]. Since the HPP affects the bacterial membrane properties [64–66], the involvement of methionine in lipid biosynthesis could play a role in the HPP resistance (Figure 4). In this regard, according to the results of the fatty acid profile of *L. monocytogenes* CTC1034 (Table 1) compared with the control conditions when the pathogen was exposed to lactate and/or HPP stresses, cells tended to increase, although not significantly, the level of total branched-chain fatty acids (BCFAs, specifically *iso* and/or *anteiso* conformations of C13, C14, C15, C16, C17). This finding agrees with the fact that in *L. monocytogenes* BCFAs contribute to membrane fluidity and resistance against environmental stresses [67].

SAM was reported to be required for the synthesis of phosphatidylcholine from phosphatidylethanolamine [68] and to have a role in transferring a methylene group to mature phospholipids that lead to the formation of cyclopropane fatty acids (CFAs), a major component of the phospholipids of the bacterial membrane bilayers [69]. A higher proportion of CFAs in the membrane bilayer of *Escherichia coli* has been shown to increase the resistance of the pathogen submitted to HPP of 500 MPa for 5 to 30 min [70]. Since the pressure resistance of *E. coli* is reported to be related to an altered membrane functionality and with the resistance of this pathogen to oxidative stress [71], it was suggested by Chen et al. [70] that CFAs could contribute to pressure resistance by increasing the resistance of membrane lipids to the oxidative stress derived from the application of the HPP. Therefore, the results of the present study point out that the exposure of *L. monocytogenes* cells to lactate prior the HPP would upregulate the methionine biosynthesis pathway, thus contributing to enhance the resistance against HPP by changes in the lipidic membrane functionality.

The higher expression of the methionine biosynthesis pathway by *L. monocytogenes* exposed to lactate and the inhibition of the biosynthesis of this amino acid by acetate reported for *E. coli* [50] could be the reason why the piezo-protective effect on *L. monocytogenes* treated at 400 MPa for 10 min was only seen for cooked ham formulated with lactate and not with diacetate [10]. Further studies regarding *L. monocytogenes* membrane functionality (membrane composition, fluidity, and integrity) as a function of the exposure of lactate and the application of the HPP need to be conducted to experimentally to confirm the role of the membrane properties on the piezo-protective effect exerted by lactate on HPP inactivation of *L. monocytogenes*.

Table 1. Fatty acid profile (mean % ± standard deviation) of *L. monocytogenes* CTC1034 after exposure of cells to lactate, after the application of the HPP (400 MPa for 10 min), and after the application of both stresses compared to control conditions (exposed to CHMM without lactate).

Fatty Acid	Condition			
	Control	Lactate	HPP	Lactate + HPP
C10:0	0.02 ± 0.03	0.09 ± 0.12	0.03 ± 0.04	0.08 ± 0.01
C12:0	1.03 ± 0.32	0.87 ± 0.07	0.73 ± 0.08	0.77 ± 0.03
C13 *iso*	0.12 ± 0.01	0.12 ± 0.10	0.06 ± 0.00	0.06 ± 0.03
C13 *anteiso*	0.23 ± 0.08	0.27 ± 0.06	0.26 ± 0.07	0.26 ± 0.06
C14 *iso*	1.26 ± 0.01	1.22 ± 0.05	1.22 ± 0.15	1.21 ± 0.16
C14	4.59 ± 0.40	3.99 ± 0.61	4.04 ± 0.77	3.99 ± 0.89
C15 *iso*	14.05 ± 0.83	15.45 ± 0.09	14.31 ± 0.28	14.59 ± 0.18
C15 *anteiso*	39.72 ± 3.06	41.78 ± 1.28	41.24 ± 0.31	41.14 ± 0.45
C15	0.42 ± 0.18	0.49 ± 0.06	0.51 ± 0.05	0.51 ± 0.12
C16 *iso*	3.13 ± 0.03	3.26 ± 0.35	3.60 ± 0.58	3.40 ± 0.21
C16	5.90 ± 2.51	4.17 ± 0.38	4.29 ± 0.60	4.03 ± 0.15
C16:1	2.62 ± 0.00	2.44 ± 1.19	2.79 ± 0.86	2.63 ± 0.58
C17 *iso*	4.63 ± 0.35	4.82 ± 0.05	5.23 ± 0.07	5.06 ± 0.20
C17 *anteiso*	16.75 ± 1.39	17.28 ± 0.60	17.91 ± 0.73	18.10 ± 0.53
C18	1.72 ± 0.71	1.11 ± 0.11	1.12 ± 0.13	1.38 ± 0.05
C18:1 cis9	3.31 ± 1.28	2.33 ± 0.10	2.40 ± 0.21	2.39 ± 0.17
C18:1 cis11	0.02 ± 0.03	0.04 ± 0.05	0.01 ± 0.02	0.00 ± 0.00
C19:0	0.11 ± 0.04	0.08 ± 0.01	0.09 ± 0.01	0.26 ± 0.10
C18:2	0.36 ± 0.17	0.20 ± 0.09	0.17 ± 0.14	0.16 ± 0.10
BCFA [a]	79.89 ± 5.67	84.20 ± 1.57	83.82 ± 0.59	83.82 ± 0.24
iso BCFA	23.19 ± 1.15	24.88 ± 0.36	24.41 ± 0.52	24.31 ± 0.37
anteiso BCFA	56.70 ± 4.53	59.32 ± 1.93	59.41 ± 1.12	59.50 ± 0.13
iso/*anteiso*	0.41 ± 0.02	0.42 ± 0.02	0.41 ± 0.02	0.41 ± 0.01
C13 BCFA	0.35 ± 0.07	0.39 ± 0.04	0.32 ± 0.08	0.32 ± 0.02
C15 BCFA	53.77 ± 3.89	57.23 ± 1.37	55.55 ± 0.59	55.73 ± 0.63
C17 BCFA	21.38 ± 1.74	22.10 ± 0.65	23.14 ± 0.66	23.16 ± 0.73
C15 BCFA/C17 BCFA	2.51 ± 0.02	2.59 ± 0.01	2.40 ± 0.05	2.41 ± 0.10
C15 *anteiso*/C17 *anteiso*	2.37 ± 0.01	2.42 ± 0.01	2.31 ± 0.08	2.27 ± 0.09

[a]—Branched-chain fatty acids.

The increased expression of the methionine pathway by *L. monocytogenes* CTC1034 under HPP stress could explain, at least partially, the piezo-stimulation effect (which was enhanced by the presence of lactate) in the growth rate of *L. monocytogenes* CTC1034 cells surviving a HPP at 600 MPa for 3 min observed by Bover-Cid et al. [11]. Since methionine is a key amino acid involved in enabling cell proliferation as precursor of anabolic pathways [72], the upregulation of the methionine biosynthesis due to lactate and HPP stresses could help *L. monocytogenes* cells to repair cellular membrane and enhance their subsequent proliferation. Nevertheless, further studies should be conducted to complement and support this.

4. Conclusions

New insights are provided regarding the molecular mechanisms underlying the protective effect of lactate on *L. monocytogenes* submitted to HPP. The short exposure of *L. monocytogenes* cells to lactate promoted a shift in the pathogen's central metabolism, favoring the propanediol and ethanolamine pathways together with the synthesis of the B12 cofactor, which could confer a competitive advantage for *L. monocytogenes* to overcome the stress suffered by HPP. Changes to the central metabolism, together with responses involving the modification of the intracellular pool of anions or pH homeostasis such as glutamate metabolism or enrichment of flagellar assembly pathways could constitute mechanisms responsible for the piezo-protective effect of lactate. The upregulation of the methionine synthesis pathway after exposure to lactate could also be relevant in relation to the piezo-resistance mechanisms through changes in the properties of the cytoplasmic

membrane and its ability to cope with pressure stress. Further studies regarding the *L. monocytogenes* membrane functionality (membrane composition, fluidity, and integrity) as a function of the exposure of lactate and the application of the HPP need to be conducted to experimentally confirm the role of the membrane properties on the piezo-protection and piezo-stimulation effect exerted by lactate on HPP inactivation of *L. monocytogenes*.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/biom11050677/s1, Table S1: Number of raw and clean reads from the transcriptomic analysis of both *L. monocytogenes* strains CTC1034 and EGDe in CHMM without and with lactate and/or without and with HPP of 400 MPa for 10 min, Table S2: List of KEGG Orthology (KO) genes differentially (FDR < 0.05) expressed in the *L. monocytogenes* strain CTC1034 in samples without lactate pressurized and non-pressurized. Positive Log2 fold change indicates genes more abundant in pressurized samples, Table S3: List of KEGG Orthology (KO) genes differentially (FDR < 0.05) expressed in the *L. monocytogenes* CTC1034 strain in non-pressurized samples without and with lactate. Positive Log2 fold change indicates genes more abundant in samples with lactate, Table S4: List of KEGG Orthology (KO) genes differentially (FDR < 0.05) expressed in the *L. monocytogenes* strain CTC1034 in pressurized samples without and with lactate. Negative Log2 fold change indicates genes less abundant in samples with lactate, Table S5: List of KEGG Orthology (KO) genes differentially (FDR < 0.05) expressed in the *L. monocytogenes* strain CTC1034 in samples with lactate non-pressurized and pressurized. Positive Log2 fold change indicates genes more abundant in pressurized samples, Table S6: List of KEGG Orthology (KO) genes differentially (FDR < 0.05) expressed in CTC1034 *L. monocytogenes* strain throughout the comparison of control samples (non-exposed to lactate and non-pressurized) to samples exposed to lactate and pressurized. Positive Log2 fold change indicates genes more abundant in samples exposed to lactate and pressurized, Table S7: List of KEGG Orthology (KO) genes differentially (FDR < 0.05) expressed in the *L. monocytogenes* strain EGDe in samples without lactate pressurized and non-pressurized. Positive Log2 fold change indicates genes more abundant in pressurized samples, Table S8: List of KEGG Orthology (KO) genes differentially (FDR < 0.05) expressed in the *L. monocytogenes* strain EGDe in pressurized samples without and with lactate. Positive Log2 fold change indicates genes more abundant in samples with lactate, Table S9: List of KEGG Orthology (KO) genes differentially (FDR < 0.05) expressed in the *L. monocytogenes* strain EGDe in samples with lactate non-pressurized and pressurized. Positive Log2 fold change indicates genes more abundant in pressurized samples, Table S10: List of KEGG Orthology (KO) genes differentially (FDR < 0.05) expressed in EGDe *L. monocytogenes* strain throughout the comparison of control samples (non-exposed to lactate and non-pressurized) to samples exposed to lactate and pressurized. Positive Log2 fold change indicates genes more abundant in samples exposed to lactate and pressurized.

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Article 6

New insights on *Listeria monocytogenes* growth in pressurized cooked ham: A piezo stimulation effect enhanced by organic acids during storage

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New insights on *Listeria monocytogenes* growth in pressurised cooked ham: a piezo-stimulation effect enhanced by organic acids during storage

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Abstract

The aim of the present study was to understand growth and survival responses of *Listeria monocytogenes* during the storage of high pressure processed (HPP) cooked ham formulated with organic acids to inhibit growth of the pathogen. Cooked ham batches were manufactured without organic acids (control), with potassium lactate (2.8% or 4%) or with potassium lactate and sodium diacetate (2.0% + 0.11% or 2.0% + 0.45%). Products were aseptically sliced and inoculated with 10^7 cfu/g or 10^2 cfu/g of either *L. monocytogenes* CTC1034 (a meat isolate) or a cocktail of three isolates (12MOB045Lm, 12MOB089Lm and Scott A). Vacuum-packed samples with 10^7 cfu/g were HPP at 600 MPa for 3 min, whereas samples with 10^2 cfu/g were not HPP. Growth or survival of *L. monocytogenes* was determined during subsequent storage at 8, 12 and 20 °C. Growth or survival was characterized by fitting the experimental data using the primary logistic model and the log-linear with shoulder model, respectively. Secondary models were fitted to characterize the effect of temperature on growth kinetic parameters without or with HPP. For cooked ham without organic acids, growth rates of *L. monocytogenes* were slightly increased by HPP and lag times were longer. Interestingly, for cooked ham with organic acids, the HPP had a significant stimulating effect on subsequent growth of *L. monocytogenes* (piezo-stimulation). At 20 °C, the growth rates of *L. monocytogenes* in cooked ham with lactate were up to 4-fold higher than those of the same product without HPP. The observed enhancement of the piezo-stimulating effect of organic acids on growth rates during storage of HPP cooked ham represents a challenge for the use of organic acids as antimicrobials in these products. A predictive model available as part of the Food Spoilage and Safety Predictor (FSSP) software seemed useful to predict growth and growth boundary of *L. monocytogenes* in non-pressurised cooked ham. This model was calibrated to take into account the observed piezo-stimulating effect and to predict growth of *L. monocytogenes* in HPP cooked ham with organic acids.

Keywords

High pressure processing; Food safety; Deli meat products; Post-lethality treatments; Safe shelf-life

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1 Introduction

High pressure processing (HPP) is a non-thermal technology with increasing application in the food industry. HPP can extend the shelf-life of perishable food while ensuring food safety (Rendueles et al., 2011). HPP is particularly interesting as a post-lethality treatment (PLT) for ready-to-eat (RTE) foods that are exposed to microbial contamination after thermal treatments such as cooked meat products commercialised in convenience format (i.e. diced, sliced and packaged). HPP causes microbial inactivation not only of spoilage microorganisms but also of pathogens like *Listeria monocytogenes*, the most relevant foodborne pathogen for this type of products (Buchanan et al., 2017).
The *Listeria* zero tolerance followed by countries such as USA forces food manufacturers to design specific risk mitigation strategies. In this framework, the Listeria rule (FSIS, 2014) rate the RTE food manufacturers according to the RTE product risk. The safest operating procedures are those validated as Alternative 1, in which PLT aiming to reduce pathogen loads are combined with antimicrobial agents (AMA) to inhibit the pathogen growth during the product shelf-life. The so called Alternative 2 consists in either the application of a PLT (Alternative 2a) or an AMA (Alternative 2b, considered as a higher risk than alternative 2a). While, the highest risk occurs when operating procedures rely exclusively on sanitation and good manufacturing practices (i.e. Alternative 3). Among AMA, organic acids and their salts (lactate, acetate, diacetate) are food additives frequently used as *L. monocytogenes* growth inhibitors in cooked meat products (Pérez-Rodríguez et al., 2017).
The effectiveness of the specific strategies needs to be validated (FSIS, 2013; 2014). In case of HPP, the microbial inactivation during processing is of primary importance. This inactivation is influenced by processing parameters such as pressure, time and temperature as well as by product characteristics that may favour lethality or protect microorganisms during HPP (Hereu et al., 2012; Rendueles et al., 2011). Therefore, the validation should be carried out through a product-oriented approach (Hereu et al., 2014). Moreover, the potential occurrence of resistant cells after HPP makes it necessary to take into consideration the behaviour of surviving bacteria, as for example cooked ham and refrigerated storage may offer conditions enabling the recovery and subsequent growth of *L. monocytogenes* during the product shelf-life (Jofré and Serra, 2016). The effect of organic acids and their salts have been extensively studied and several predictive tools can be used to design products not supporting the growth of the pathogen (Mejlholm et al., 2010). However, scarce information is available about the possible interaction between HPP and antimicrobials, particularly organic acids and their salts. Based on the antimicrobial hurdle concept (Leistner, 2007), an additive or a synergistic effect may be expected. However, in previous studies with cooked ham an increased HPP resistance of *L. monocytogenes* was observed by the presence of lactate in the product formulation but the subsequent growth or survival of the pathogen during the product shelf-life was not studied (Bover-Cid et al., 2016).
The present work was carried out to study the behaviour of *L. monocytogenes* during storage of HPP cooked ham formulated without or with natural antimicrobials often used by the meat industry, i.e. potassium lactate (E-326) and sodium diacetate (E-262). The *L. monocytogenes* growth and growth-boundary model included in the Food Spoilage and Safety Predictor (FSSP, v4.0) was used to design experiments where some formulations were close to the growth boundary of the pathogen. This allowed the combined effect of HPP and organic acids to be studied close the growth boundary, which is important as products stabilized against growth of *L. monocytogenes* are desirable.

2 Material and methods

2.1 Cooked ham manufacture and characterization

Cooked ham was manufactured *ad-hoc* using pork meat and the following ingredients (g/kg): water, 120; salt, 20.7; sodium tripolyphosphate, 5.8; dextrose, 5.8; carragenate, 2.3; sodium ascorbate, 0.6; and sodium nitrite 0.1. For the 34 combinations of conditions studied (see 2.3) five different batches were manufactured, one without organic acids as control product, two with potassium lactate (HiPure Corbion®, Montmeló, Spain) at 2.8% and 4.0% and two with potassium lactate and sodium diacetate (Grama Aliment SL, Les Preses, Spain) at 2% + 0.11% or 2% + 0.45%. The concentrations of potassium lactate and sodium diacetate were selected close to the growth/no growth boundary for *L. monocytogenes* at 8 °C and 12 °C, respectively, according to the predictive model "Growth of *Listeria monocytogenes* in chilled seafood and meat products" available in the Food Spoilage and Safety Predictor (FSSP v4.0) software (http://fssp.food.dtu.dk). Meat was minced in a cutter to a particle size of 6 mm. Ingredients were

homogenized in a mixer for 30 min, stuffed into an impermeable plastic film, and cooked in an oven at 68 °C for 5 h resulting in a product core temperature of 65 °C. For each formulation up to five blocks of *ca*. 3 kg each were manufactured.
Product a_w was measured with an AquaLab™ instrument (Series 3; Decagon Devices Inc., Pullman, WA, USA). pH was measured by direct measurement with a penetration probe (52-32; Crison Instruments SA, Alella, Spain) connected to a portable pH-metre (PH 25; Crison Instruments). Concentrations of organic acids were determined from an acid extract of a cooked ham sample by HPLC, using an ion exclusion column (Transgenomic ICSepICE-ORH-801, Chrom Tech. Inc., MN, USA) with a refractive index (RI) detector. Nitrites were determined by spectrofluorometry and sodium by flame atomic absorption spectroscopy according to the Spanish official methods (Anonymous, 1979). The fat, protein and water contents were determined according to the AOAC official method 2007.04 (Anderson, 2007) with a FoodScan™ device (FOSS, Hillerød, Denmark).

2.2 L. monocytogenes strains and pre-culture conditions

Strains of *L. monocytogenes* used in the present study included: the meat isolate CTC1034 (serotype 4b) from the IRTA culture collection and previously used in our studies dealing with HPP meat products (Bover-Cid et al., 2015; 2011; Hereu et al., 2012a; Hereu et al., 2012b; Hereu et al, 2014); the reference strains 12MOB045LM (genoserotype II) and 12MOB089LM (genoserotype IV) from the European Reference Laboratory for *L. monocytogenes*, both recommended for challenge tests with meat products (EURL Lm, 2014); and Scott A (4b) a clinical isolate frequently included in HPP inactivation studies (van Boeijen et al., 2008).
Strains were kept at −80 °C in Brain Heart Infusion (BHI) broth (Beckon Dickinson, Sparks, Md., USA) with 20% glycerol until used. These conditions provided slightly more pressure resistant cells (conservative approach) than pre-culturing at refrigeration temperatures, though without modifying the growth rate of the pathogen during the subsequent growth (Hereu et al., 2014). Thawed cultures of the strain CTC1034 were directly used to inoculate cooked ham slices at *ca*. 10^7 cfu/g or were diluted to 10^5 cfu/g with physiological saline (0.85% NaCl and 0.1% Bacto Peptone) to inoculate cooked ham slices at *ca*. 10^2 cfu/g (see section 2.3). A cocktail including 12MOB045LM, 12MOB089LM and Scott A (Lm-mix) was prepared by mixing the respective thawed cultures at equal concentrations before being directly inoculated (1% v/w) to the products or diluted as described for the CTC1034 strain.

2.3 Challenge tests, HPP and storage conditions

Cooked hams with the five different formulations (see section 2.1) were sliced in the laboratory under aseptic conditions. Slices of each type of cooked ham were surface spiked with either the *L. monocytogenes* CTC1034 strain or with the cocktail of three strains (i.e. 12MOB045LM, 12MOB089LM and Scott A). This inoculation was performed by using a laminar flow cabinet to avoid contamination with other microorganisms. The inoculum level for either the single strain or the mix of strains was 1% (v/w) to reach a final concentration of *ca*. 10^7 cfu/g for products to be HPP and *ca*. 10^2 cfu/g for non-pressurised products. These different inoculum levels between HPP and non-pressurised products were necessary to enable quantitative characterization of the growth curve. The inoculated volume was spread on the whole surface of the ham slices with a single-use sterile Digralsky spreader and then let to be adsorbed for 2 min. under a laminar flow of sterile air. Inoculated slices of each product were vacuum packaged (EV-15-2-CD; Tecnotrip, Terrassa, Spain) in PET/PE bags (oxygen permeability < 50 cm^3/m^2/24 h and low water vapour permeability < 15 mg/m^2/24 h; Sacoliva S.L., Barcelona, Spain). Samples were pressurised at 600 MPa for 3 min using commercial high pressure processing equipment (Wave 6000; Hiperbaric, Burgos, Spain) at an initial water temperature of 15 °C. The come-up rate was on average 220 MPa/min and the pressure release almost instantaneous (< 6 s). Samples inoculated with the lower inoculum were not pressure treated and used as controls. Pressurised and non-pressurised samples were stored at 8 and 12 °C for 16 to 90 days. These temperatures are recommended by the European Reference Laboratory of *L. monocytogenes* Guidelines to conduct challenge test to study the safe-shelf life of ready-to-eat food (EURL Lm, 2014). Furthermore, for products inoculated with *L. monocytogenes* strain CTC1034 storage at 20 °C during 10 to 58 days was also studied to better characterize the effect of HPP and organic acids on growth of the pathogen. A total of 34 experimental conditions combining product formulation, *L. monocytogenes* strains, storage temperatures and HPP were studied (see Tables 1 and 2).

2.4 Microbiological analysis

To monitor *L. monocytogenes* growth behaviour, samples from all 34 experimental conditions in the study were periodically analysed with a total of 30 to 44 data points distributed all along the storage period. Each sample was homogenized 1/10 in a bag Blender Smasher® (bioMérieux, Marcy-l'Étoile, France) and 10-fold serially diluted in physiological saline (0.85% NaCl and 0.1% Bacto Peptone). Enumeration of *L. monocytogenes* was performed on the CHROMagar™ *Listeria* chromogenic media (CHROMagar, Paris, France) incubated at 37 °C for 24 h. To achieve a quantification limit of 2 cfu/g, 5 ml of the 1/10 diluted homogenate was pour plated into plates with a diameter of 14 cm. For samples with expected concentration of *L. monocytogenes* below this quantification limit, the presence/absence of the pathogen was investigated by enrichment of 25 g-samples in 225 ml tryptic soy broth (Becton Dickinson) supplemented with 0.6% yeast extract (TSBYE) and incubated for 48 h at 37 °C. After enrichment, the presence of *L. monocytogenes* was detected by plating on CHROMagar™ *Listeria*. For modelling purposes, absence in 25 g was computed as -1 Log cfu/g, presence below the quantification was computed as -0.3 Log cfu/g.
Additionally, the potential contamination by lactic acid bacteria (LAB) in cooked ham samples (both pressurised and non-pressurised) was checked along the experiments by plating the homogenized 1/10 dilution into MRS (de Man Rogosa and Shape) agar plates (Merck), which were incubated at 30 °C for 72h under anaerobiosis. For the experiments carried out with the control ham at 8 °C, a high sampling frequency was carried out (i.e. on days 0, 1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 14, 15 and 17). No LAB were detected and this is likely related to the aseptic conditions applied during cooked ham manipulation (slicing and packaging). For the other trials the absence of LAB (<10 cfu/g) was verified occasionally with 3 to 4 sampling times along the storage time.

2.5 Primary growth modelling

To estimate the kinetic growth parameters for each growth curve, the primary Logistic growth models with delay ($\lambda > 0$) and without delay ($\lambda = 0$) (Eq. (1), (Rosso et al., 1996)) were fitted to the log-transformed counts using the nls2 and nls function form the respective nls2 and nls packages of R (R Core Team, 2013).

$$\text{If } t < \lambda \;\; \text{Log}(N_t) = \text{Log}(N_0)$$
$$\text{If } t \geq \lambda \;\; \text{Log}(N_t) = \text{Log}\left(\frac{N_{max}}{1+\left(\frac{N_{max}}{N_0}-1\right)*\left(\exp\left(-\mu_{max}*(t-\lambda)\right)\right)}\right) \quad \text{Eq. (1)}$$

Where *t* is time (d); N_0 is the bacterial concentration (cfu/g) at time zero; N_t is the bacterial concentration (cfu/g) at time *t*, N_{max} is the maximum bacterial concentration (cfu/g), λ is the lag time (d) and μ_{max} is the maximum specific growth rate (d^{-1}).
The *F*-test was applied to determine the statistical significance of the estimated lag time for each growth curve (Dalgaard, 1995).

For the combination of conditions not supporting growth and compromising the viability of the pathogen a log-linear with shoulder primary model (Eq. (2), (Geeraerd et al., 2000)) was fitted to the data.

$$\text{If } t \leq S;$$
$$\text{Log}(N) = \text{Log}(N_0)$$
$$\text{If } t > S;$$
$$\text{Log}(N) = \text{Log}(N_0) - \left(\frac{k_{max}*t}{\text{Ln }(10)}\right) + \text{Log}\left(\frac{\exp^{(k_{max}*S)}}{1+[\exp^{(k_{max}*S)}-1]*\exp^{(-k_{max}*t)}}\right) \quad \text{Eq. (2)}$$

Where *t* is time (d); N_0 is the bacterial concentration (cfu/g) at time zero, k_{max} is the maximum specific inactivation rate (d^{-1}) and *S* is the shoulder (d). The *F*-test was applied to determine the statistical significance of the shoulder for each growth curve.

2.6 Comparison of observed and predicted growth rates

The growth rates observed at different combination of experimental conditions were compared with those predicted by the model of Mejlholm and Dalgaard (2009) available in the Food Spoilage and Safety Predictor

(FSSP v4.0) as "Growth of *Listeria monocytogenes* in chilled seafood and meat products". This model was previously found suitable to predict the growth behaviour of *L. monocytogenes* in cooked ham (Mejlholm et al., 2010). The comparison was performed to facilitate a quantitative evaluation of effects by experimental condition rather than as an evaluation of the specific predictive model. Growth was predicted by taking into account storage temperature and product characteristics for each experimental condition (see Table 3). Observed and predicted growth was compared by calculation of bias- (B_f) and accuracy (A_f) factors for the μ_{max}–values (Dalgaard and Jorgensen, 1998). The bias factor values were calculated so that numbers lower than 1 always indicated that predicted growth was slower than observed growth. As an example, a B_f-value of 0.75 indicates predicted growth rates to be 25% slower than observed growth rates (Mejlholm et al., 2010; Ross, 1996). A_f-values > 1.5 have previously been shown to indicate incomplete models or systematic deviation between observed and predicted μ_{max}-values (Mejlholm and Dalgaard, 2013).

2.7 Secondary growth modelling

Secondary modelling was applied to assess the effect of the storage temperature on the primary growth parameters (μ_{max}, λ and N_{max}) of *L. monocytogenes* in cooked ham without added organic acids. The modified Ratkowsky square root model (Eq. (3); (Ross and Dalgaard, 2004)) was used to fit the growth rate (μ_{max}, d^{-1}) values determined at different storage temperatures.

$$\sqrt{\mu} = \sqrt{\mu_{ref}} \cdot \left(\frac{T - T_{min}}{T_{ref} - T_{min}}\right) \quad \text{Eq.(3)}$$

Where μ_{ref} is the estimated growth rate (d^{-1}) at a reference temperature, T_{ref} is the temperature of reference fixed at 25 °C (Mejlholm and Dalgaard, 2009), and T_{min} is the estimated theoretical minimum temperature for *L. monocytogenes* growth. The relative lag time (RLT) concept, defined as the ratio of the lag time to the generation time (GT = Ln(2)/μ_{max}) was used to develop a secondary lag time (λ) model (Eq. (4), (Ross and Dalgaard, 2004)). Where a potential effect of storage temperature on RLT was modelled as previously described (Hereu et al., 2014) with the parameters k_0 and k_1 characterizing a potential temperature dependance of RLT.

$$\lambda = RLT \cdot \frac{\mathrm{Ln}(2)}{\mu_{max}} = k_0 + \frac{k_1}{T^2} \cdot \frac{\mathrm{Ln}(2)}{\mu_{max}} \quad \text{Eq. (4)}$$

The effect of storage temperature on log (N_{max}) was described by using a simple linear equation (Eq. (5)) where *a* is log (N_{max}) at 0°C and b a slope parameter.

$$\mathrm{Log}(N_{max}) = a + b \cdot T \quad \text{and} \quad N_{max} = 10^{(a + b \cdot T)} \quad \text{Eq. (5)}$$

Following the two-step modelling approach, a one-step or global regression procedure was applied. A global model (Eq. (6)) integrating the primary model (Eq. (1)) and the secondary models for λ, μ_{max} and N_{max} was fitted to the data set with 350 Log cfu/g values for cooked ham without added organic acids. The *F*-test was applied to assess the need of two different models for non-HPP and HPP products. The goodness of fit of the developed models was assessed by means of residual sum of square (RSS), root mean square error (RMSE) and determination coefficients (R^2 and R^2_{adj}).

If $t < \lambda$

$$\mathrm{Log}(N_t) = \mathrm{Log}(N_0) \qquad \text{Eq. (6)}$$

If $t \geq \lambda$

$$\mathrm{Log}(N_t) = \mathrm{Log}\left(\frac{10^{(a+b\cdot T)}}{1+\left(\frac{10^{(a+b\cdot T)}}{N_0}-1\right)\cdot\left(\exp\left(-\left(\mu_{ref}\cdot\left(\frac{\mathrm{T}-T_{min}}{\mathrm{T_{ref}}-T_{min}}\right)^2\right)\cdot\left(t-\left(k_0+\frac{k_1}{T^2}\cdot\frac{\mathrm{Ln}(2)}{\mu_{ref}\cdot\left(\frac{\mathrm{T}-T_{min}}{\mathrm{T_{ref}}-T_{min}}\right)^2}\right)\right)\right)\right)}\right)$$

3 Results and discussion

3.1 Listeria monocytogenes behaviour in non-pressurised cooked ham

Growth and survival responses of *L. monocytogenes* in 17 challenge tests for cooked ham without HPP are shown in Fig. 1 (empty symbols) with fitted kinetic parameters from primary models shown in Table 1.

Table 1 Estimated parameter values resulting from fitting the primary kinetic models to the *L. monocytogenes* counts on cooked ham not pressurised.

Experimental conditions				Kinetic parameters					Goodness of fit[c]			
Temperature (°C)	Added lactate (%)	Added diacetate (%)	Strain	G[a] I[b]	Log N_0 (Log cfu/g)[a,b]	λ (d)[a] s (d)[b]	μ_{max} (d^{-1})[a] $-k_{max}$ (d^{-1})[b]	Log N_{max} (Log cfu/g)[a]	n	RSS	RMSE	R^2_{adj}
8	-	-	CTC1034	G	2.5	0.5	1.010	8.4	38	1.77	0.23	0.988
8	-	-	Mix	G	2.6	1.6	1.020	8.0	38	1.88	0.24	0.986
8	2.8	-	CTC1034	G	2.2	43.9	0.460	5.9	38	30.02	0.94	0.743
8	2.8	-	Mix	G	2.4	50.4	0.300	5.8	38	23.50	0.83	0.694
8	2.0	0.11	CTC1034	I	2.4	0.0	-0.001	-	38	4.71	0.37	0.253
8	2.0	0.11	Mix	I	2.3	63.1	-0.069	-	38	0.82	0.15	0.750
12	-	-	CTC1034	G	2.7	0.6	2.046	8.6	33	1.14	0.20	0.993
12	-	-	Mix	G	2.4	0.7	1.842	8.3	33	0.95	0.18	0.994
12	4.0	-	CTC1034	I	2.8	19.0	-0.145	-	35	6.61	0.46	0.904
12	4.0	-	Mix	I	2.6	7.0	-0.038	-	35	6.57	0.45	0.455
12	2.0	0.45	CTC1034	I	2.6	21.4	-0.143	-	31	11.62	0.64	0.820
12	2.0	0.45	Mix	I	2.4	28.1	-0.133	-	31	6.45	0.48	0.846
20	-	-	CTC1034	G	2.7	0.1	4.692	8.8	32	0.94	0.18	0.993
20	2.8	-	CTC1034	G	2.5	1.8	1.503	7.1	32	0.48	0.13	0.996
20	4.0	-	CTC1034	G	2.6	4.7	0.571	6.9	42	1.64	0.21	0.984
20	2.0	0.11	CTC1034	G	2.6	2.2	0.712	7.0	39	2.15	0.25	0.982
20	2.0	0.45	CTC1034	I	2.5	7.7	-0.190	-	44	9.99	0.49	0.854

[a] G, for conditions supporting growth the logistic with delay model was fitted to the data (Eq. (1)) to estimate the kinetic parameters Log N_0: initial bacterial concentration; λ: lag time; μ_{max}: maximum specific growth rate; Log N_{max}: maximum bacterial concentration.

[b] I, when conditions not supporting growth caused a loss of *L. monocytogenes* viability (i.e. inactivation), the log-linear with shoulder model was fitted to the data (Eq. (2)) to estimate the kinetic parameters Log N_0: initial bacterial concentration; S: shoulder; k_{max}: inactivation rate; Log N_{max}: maximum bacterial concentration.

[c] n: number of data (cell concentrations, Log cfu/g) included for fitting; RSS: residual sum of squares; R^2_{adj}: adjusted coefficient of determination. Values obtained for experiments at each combination of conditions

As expected, growth of the pathogen was observed for non-pressurised cooked ham formulated without organic acids (Fig. 1a, b and c). Similar growth curves were found for *L. monocytogenes* CTC1034 and for the mix including the reference strains 12MOB045LM, 12MOB089LM and Scott A (Fig. 1). The expected prevention of growth due to added organic acids was found for products stored at 8, 12 and 20 °C (Fig. 1h, I and k, empty symbols). Under these conditions, the viability of *L. monocytogenes* was compromised. A log-linear decreasing trend was observed, with k_{max} as maximum specific inactivation rate, after surviving for some time with the shoulder parameter being statistically significant ($p < 0.05$) in most survival curves (survival parameter estimates are also included in Table 1). The addition of 2.8% lactate in non-pressurised cooked ham (Fig. 1d, empty symbols) extended the lag time in comparison with the control without lactate, but it did not prevent growth of *L. monocytogenes*. In this case, the wide dispersion of the observed levels of *L. monocytogenes* along the storage made the estimation of growth kinetic parameters more uncertain than in the control products, as indicated by the goodness of fit parameters (Table 1). At 20 °C, *L. monocytogenes* was able to grow in the presence of 4% lactate and with a combination of 2% lactate plus 0.11% diacetate (Fig. 1g and j), although these conditions prevented growth at 8 °C (Fig. 1h) and 12 °C (Fig. 1e). As expected, lag times were longer and growth rates lower compared to control product without organic acids (Table 1). With 2% lactate plus 0.45% diacetate inactivation of *L. monocytogenes* was observed for non-pressurised cooked ham at 12 °C and inactivation was faster at 20 °C (Fig. 1k; Table 1). The faster inactivation at a higher temperature under growth-preventing conditions (Fig. 1i and k) is in agreement with previous studies of both *L. monocytogenes* and *E. coli* (Ross et al., 2008; Zhang et al., 2010). LAB were not detected (i.e. < 10 cfu/g) in any of the samples analysed along the experiments, therefore *L. monocytogenes* behaviour was not determined by the interaction with endogenous LAB.

For cooked ham non-HPP growth responses were in accordance with those predicted by the FSSP model without LAB interaction, as shown by the B_f -value of 0.89 indicating that growth rates on average were predicted to be 11% slower that observed. Without or with added organic acids the B_f -values were, respectively, 0.95 and 0.84 (Table 3). Of the 17 experimental conditions for non-pressurised products, growth or no-growth responses were correctly predicted for 15 trials, whereas for cooked ham with 4% lactate the prediction of growth with μ_{max} of 0.25 d^{-1} was fail-safe as slight inactivation was observed for both CTC1034 and Lm-mix (Fig. 1e, Table 1). This difference between observed and predicted growth may be due to minor deviations between actual and measured product characteristics. If for example the product pH actually were 6.09 rather than the measured 6.15 (Table 3) then the applied model would correctly predict no-growth for this product formulation with high lactate concentration.

3.2 Listeria monocytogenes behaviour in HPP cooked ham

The applied HPP (600 MPa for 3 min) caused a significant inactivation on *L. monocytogenes* of about 7 log units (Results not shown). Just after the HPP, *L. monocytogenes* was detected in all samples though at levels below the quantification limit in most of the samples, hampering a more precise quantification of the log reductions.

In the HPP control cooked ham (Fig. 1a, b, c, full symbols) the surviving *L. monocytogenes* cells were able to initiate growth after a relatively short time post-HPP. Without added organic acids the lag times and growth rates in HPP cooked ham (Table 2) were slightly higher than those observed for non-HPP products (Table 1). Some works have dealt with the behaviour of piezo-tolerant isolates of *L. monocytogenes* Scott A and LO28 in comparison with the wild type counterpart (Joerger et al., 2006; Karatzas and Bennik, 2002; Van Boeijen et al., 2010). In these works, *L. monocytogenes* mutants exhibited identical or slightly lower growth rate in comparison with the wild-type strain. These studies applied lower pressures (150 MPa to 500 MPa) than those used in the present work and by the meat industry nowadays. Besides, they were performed in simple laboratory media such as brain heart infusion (BHI). Under these conditions the effect of food matrix components was omitted and thus results may not be comparable with the findings of the present study carried out with meat products.

Surprisingly, *L. monocytogenes* was able to grow in HPP products formulated with organic acids at concentrations that prevented growth in non-pressurised cooked ham (Fig 1h, e, i). This was observed both for *L. monocytogenes* CTC1034 and for the mix of strains (Fig. 1). In these challenge tests, the estimated growth parameters were less accurate due to the occurrence of results below the quantification limit. The unexpected growth could result from HPP-resistant cells indicating the occurrence of a heterogeneous *L. monocytogenes* population including piezo-sensitive and piezo-resistant fractions (Hereu et al., 2014; Van Boeijen et al., 2010).

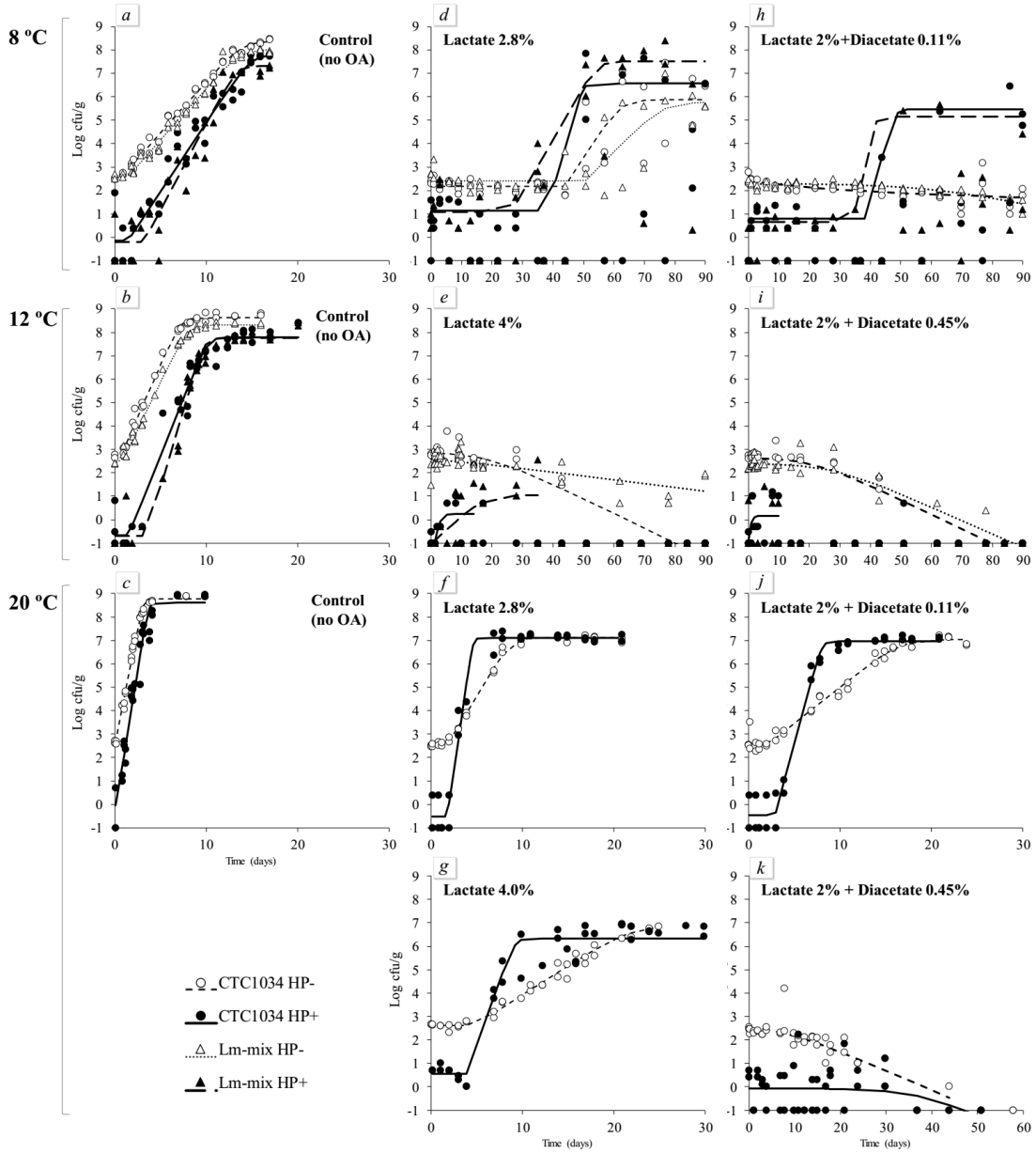

Fig 1. Growth of *L. monocytogenes* in cooked ham formulated without (control) or with organic acids and stored at 8, 12 or 20 °C. Symbols represent cell concentration (Log cfu/g) and lines the fitted data. Non-pressurised (HP-) and pressurised (HP+, 600 MPa/ 3min/15 °C) samples are represented with empty and solid symbols, respectively.

Thus, the unexpected growth could represent the behaviour of the cells that were able to resist, recover from potential sub-lethal damage and grow during the subsequent refrigerated storage of the product. However, growth rates of *L. monocytogenes* in HPP products with organic acids were markedly higher than for non-pressurised products with the same concentrations of organic acids. This was most pronounced at 20 °C where *L. monocytogenes* was able to grow up to 4-fold faster in comparison with the corresponding non-HPP conditions (Fig. 1f, g, j; Tables 1 and 2). Thus, in the presence of organic acids a pronounced piezo-stimulation by HPP was observed. The comparison of observed and predicted growth contributed to the quantification of this piezo-stimulating effect as the applied growth and growth boundary model included in the FSSP software did not take this effect into account. With HPP and without added organic acids the B_f value of 0.71 showed predicted growth rates to be 29% slower that observed, whereas with both HPP and added organic acids B_f was 0.29 and predicted growth rates were 71% slower that observed (Table 3). Of the 17 experimental conditions with HPP, growth or no-growth responses were correctly predicted for 12 (Fig.1, Table 1) and fail-dangerous predictions were obtained with 2% lactate plus 0.11% diacetate at 8°C and with 2% lactate plus 0.45% diacetate at 12°C and 20°C (Fig. 1). With 2% lactate plus 0.45% diacetate at

12 °C and at 20 °C just a few samples showed concentrations higher than those measured immediately after HPP, suggesting these conditions to be close to the growth boundary (Fig. 1i, 1k). However, the piezo-stimulating effect due to HPP and organic acids moved the growth boundary conditions.

Table 2 Estimated parameter values resulting from fitting the primary kinetic models to the *L. monocytogenes* counts on cooked ham pressurised (at 600 MPa/3 min/15 °C).

Experimental conditions				Kinetic parameters					Goodness of fit[c]			
Temperature (°C)	Added lactate (%)	Added diacetate (%)	Strain	G[a] / I[b]	Log N_0 (Log cfu/g)[a,b]	λ (d)[a] / s (d)[b]	μ_{max} (d^{-1})[a] / $-k_{max}$ (d^{-1})[b]	Log N_{max} (Log cfu/g)[a]	n	RSS	RMSE	R^2_{adj}
8	-	-	1034	G	-0.1	1.4	1.350	7.8	38	17.66	0.72	0.942
8	-	-	Mix	G	-0.2	3.2	1.700	7.3	37	26.04	0.89	0.920
8	2.8	-	1034	G	1.1	38.2	1.080	6.6	21[e]	13.60	0.89	0.899
8	2.8	-	Mix	G	1.1	26.3	0.522	7.5	26[e]	11.84	0.73	0.942
8	2.0	0.11	1034	G	0.8	38.2	1.070	5.5	16[e]	3.10	0.51	0.944
8	2.0	0.11	Mix	G	0.6	34.0	1.360	5.2	19[e]	2.27	0.39	0.949
12	-	-	1034	G	-0.7	1.5	2.236	7.8	37	13.07	0.63	0.966
12	-	-	Mix	G	-0.7	2.9	2.709	7.8	36	9.42	0.54	0.976
12	4.0	-	1034	G/NG[d]	-0.9	1.3	1.475	0.2	16[e]	10.31	0.89	0.144
12	4.0	-	Mix	G/NG[d]	-1.0	0.3	0.279	1.1	22[e]	15.71	0.91	0.400
12	2.0	0.45	1034	NG[d]	-	-	-	-	36	-	-	-
12	2.0	0.45	Mix	NG[d]	-	-	-	-	36	-	-	-
20	-	-	1034	G	-0.3	0.0	5.517	8.6	29	10.94	0.66	0.952
20	2.8	-	1034	G	-0.5	1.7	6.169	7.1	29	7.01	0.53	0.976
20	4.0	-	1034	G	0.6	3.9	2.520	6.3	35	10.23	0.57	0.953
20	2.0	0.11	1034	G	-0.5	2.9	3.300	7.0	30	5.97	0.48	0.982
20	2.0	0.45	1034	I	-0.1	36.3	-0.183	-	40	19.13	0.89	0.048

[a] For conditions supporting growth the logistic with delay model was fitted to the data (Eq. (1)) to estimate the kinetic parameters Log N_0: initial bacterial concentration after the HP treatment; λ: lag time; μ_{max}: maximum specific growth rate; Log N_{max}: maximum bacterial concentration.

[b] When conditions not supporting growth caused a loss of *L. monocytogenes* viability, the loglinear with shoulder model was fitted to the data (Eq. (2)) to estimate the kinetic parameters Log N_0: initial bacterial concentration after the HP treatment; S: shoulder; k_{max}: inactivation rate; Log N_{max}: maximum bacterial concentration.

[c] n: number of data (cell concentrations, Log cfu/g) included for fitting; RSS: residual sum of squares; R^2_{adj}: adjusted coefficient of determination. Values obtained for experiments of each combination of conditions.

[d] no clear growth (NG) or inactivation was observed.

[e] data indicating no growth (i.e. below the quantification limit) were excluded for the primary growth model fitting. Growth parameters correspond to the worse case scenario represented by recovered cells that were able to initiate growth

If organic acids are used to control *L. monocytogenes* growth in HPP cooked ham, it is very important that concentrations of these antimicrobials are sufficient to efficiently prevent growth of the pathogen. Therefore, the piezo-stimulating effect needs to be taken into account. A mathematical model and software to predict the required concentrations of organic acids or their salts depending on product characteristics, storage conditions and HPP would be most useful but to our knowledge is not available. However, for a specific HPP of 600 MPa for 3 min at 15 °C the *L. monocytogenes* growth and growth boundary model from the FSSP software can be calibrated to cooked ham with added organic acids. This is obtained by multiplying the μ_{ref}-value in the Mejlholm and Dalgaard (2009) model with a value of 3.4 corresponding to $1/B_f$ for HPP cooked ham with organic acids (Table 3) as previously reported for other cardinal parameter models (Østergaard et al., 2014; Pin et al., 1999). The calibrated model is product specific and it can be used to predict the inhibiting effect of lactate and diacetate on growth rates of *L. monocytogenes* in HPP cooked ham with added organic acids. Importantly, this model calibration does not influence the predicted growth boundary.

The observed piezo-stimulation of *L. monocytogenes* growth is unlikely to be due to differences or changes on the major physico-chemical characteristics (such as the pH, a_w, etc.) of the products as the same batch of cooked ham was used with or without HPP and no change in pH of samples was recorded after HPP. The possible effect of the amount of glycerol (0.2%) added on the matrix as a results of the inoculation with a *L. monocytogenes* culture was also considered negligible according to previous findings published in Hereu et al. (2014), where the growth of *L. monocytogenes* inoculated on cooked ham adding no glycerol, 0.0002 and 0.2% glycerol was studied in parallel. However, it cannot be excluded that HPP cause organic acids to

react with components in cooked ham and that this may reduce their antimicrobial activity. If this was the case it becomes important to test the piezo-stimulating effect in other foods. For some bacteria, the recovery after HPP is favoured under less oxidative conditions (Kimura et al., 2017). Besides the removal of oxygen by vacuum packaging, the addition of lactate and diacetate, with recognised antioxidant potential (FAO/WHO, 1995), could contribute to a better recovery, but this hardly explains the piezo-stimulation observed in the present study. To better understand the piezo-stimulating effect it seems important to determine if fast growing *L. monocytogenes* in HPP cooked with organic acids retain this growth potential after isolation from the product. Furthermore, it would be interesting to compare at genomic and transcriptomic level wild and fast growing *L. monocytogenes* isolates from HPP cooked ham with and without added organic acids. The influence of different initial fluid temperatures for HPP has been scarcely studied. In dry-cured ham, the initial fluid temperature within the rage from 7.6 to 24.4 °C had no impact on *L. monocytogenes* inactivation by HPP (Bover-Cid et al., 2011). However, the impact of this processing parameter on the subsequent growth of the pathogen, particularly in the presence of organic acids with a piezo-stimulating effect observed in the present study remains to be elucidated, and this is another point for potential future studies.

Table 3 Comparison of observed and predicted growth rates.

	n	Bias factor (B_f)	Accuracy factor (A_f)
non-HPP			
Without added acids[a]	5	0.95	1.08
With added acids[b]	5	0.84	2.05
All data[a,b]	10	0.89	1.49
HPP			
Without added acids[a]	5	0.71	1.40
With added acids[b]	7	0.29	3.42
All data[a,b]	12	0.42	2.36
Both non-HPP and HPP	22	0.58	1.97

[a] FSSP input parameters: pH = 6.07; water phase salt = 2.71%; water phase lactate (endogenous) = 7,034 ppm

[b] FSSP input parameters for cooked ham with 2.8% K-lactate: pH=6.11; water phase salt = 2.72%; water phase lactate (endogenous+added) = 34,369 ppm. For cooked ham with 4% K-lactate: pH= 6.15; water phase salt = 2.82%; water phase lactate (endogenous+added) = 45,171 ppm. For cooked ham with 2% K-lactate plus 0.11% Na-diacetate: pH 5.88; water phase salt = 2.88%; water phase lactate (endogenous+added) = 26,717 ppm; water phase diacetate = 1,247 ppm.

3.3 Secondary modelling

The secondary and global modelling was used to more precisely describe the quantitative effect of storage temperature on *L. monocytogenes* growth in cooked ham without organic acids and both without and with HPP. Fig. 2 shows the effect of storage temperature on the observed growth kinetic parameters and the fit of the secondary models for μ_{max} and λ. No significant differences ($p > 0.05$) were observed between *L. monocytogenes* CTC1034 and the mix of strains, thus data was considered together.

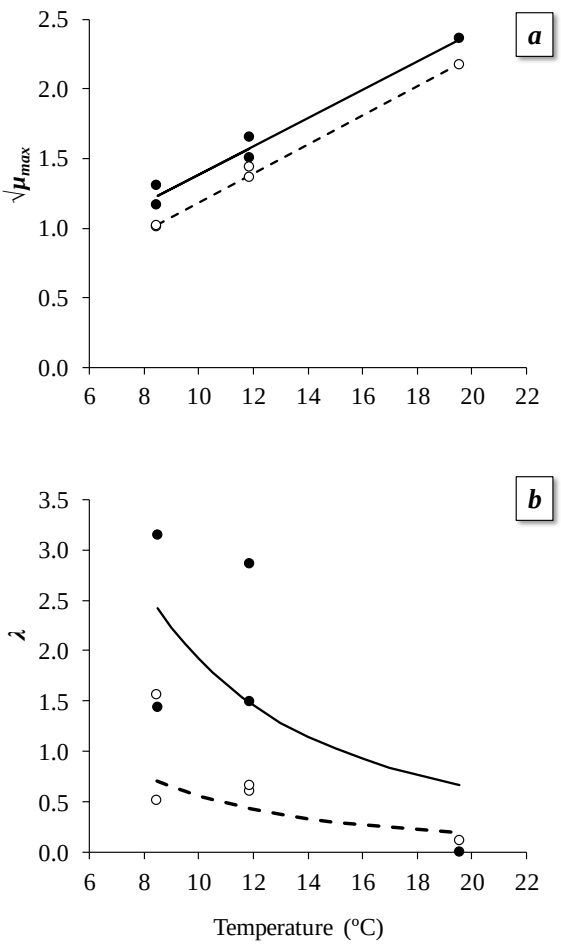

Fig 2. Effect of storage temperature on square root transformed growth rate (μ_{max}, plot *a*) and lag time (*λ, plot b*). Data and model fit for non-pressurised samples are shown with empty symbols and dashed lines, respectively. Data and model fit for pressurised samples (600 MPa/3 min/15 °C) are shown with solid symbols and continuous line, respectively. Estimated parameters values obtained with the global one-step regression are shown in Table 4.

One step fitting of the global model showed growth of *L. monocytogenes* to be statically different for products without or with HPP (p = 0.008). Growth of *L. monocytogenes* was faster in products submitted to HPP, confirming the piezo-stimulation effect in cooked ham without added organic acids. Therefore, two different secondary models were used to describe the effect of storage temperature on growth rates (Fig. 2a, Table 4).

In a previous study dealing with *L. monocytogenes* CTC1034 in cooked meat products without organic acids, HPP at 400 MPa (5 min) did not cause a significant difference on the μ_{max} in comparison with non-pressurised products (Hereu et al., 2014). In fact, the model obtained in the present work describes a very similar *L. monocytogenes* behaviour to that of the previous model build with non-pressurised and 400 MPa-treated products (Hereu et al., 2014) as well as to the behaviour predicted by the FSSP model (results not shown). This finding could suggest that higher pressure levels (i.e. 600 MPa, as applied in the present study) may be necessary to cause a detectable increased growth rate. In this line, (Jofré et al., 2008) carried out challenge tests with *L. monocytogenes* inoculated at 10^4 cfu/g in cooked ham with 1.8% potassium lactate in comparison with cooked ham (without lactate) and during the subsequent chill storage after HPP at 600 MPa (for 5 min at 10 °C), more positive samples were recorded in cooked ham with lactate compared to the control cooked ham. However, the effect of HPP on the subsequent growth rate of piezo-resistant bacteria has been scarcely studied from a quantitative perspective and the present study provides new information.

A substantial variability of lag times at the same storage temperature were observed particularly for HPP products (Fig. 2b). The observed data is in line with the previous work (Hereu et al., 2014), in which lag time of *L. monocytogenes* (previously frozen as in the present study) was extended when HPP was applied in comparison with non-HPP products (Fig. 2b, Table 4). Opposed to Hereu et al. (2014) fitting of the global model and *F*-testing showed RLT-values to be independent of the storage temperature (i.e. K_1 = 0, Table 4).

Lag time extension due to HPP can be related to the time taken by *L. monocytogenes* cells to recover from the sub-lethal damage caused by HPP before growth is initiated i.e. physiological lag. However, HPP may also create fractions of growing and non-growing cells that contribute to the observed population lag time (Hereu et al., 2014; Koutsoumanis, 2008).

Table 4. Parameter estimates of global regression model (Eq.(6)) for the growth of *L. monocytogenes* in cooked ham formulated without organic acid salts, obtained for two data sets (from non-pressurised and pressurised products).

	Growth rate model parameters		Lag time model parameter	Maximum population density parameter		Goodness of fit			
	μ_{ref} (d^{-1})	T_{min} (°C)	k_0[a]	a[b]	b	RSS	RMSE	R^2	R^2_{adj}
Non-HPP	7.958	-0.644	1.49	7.88	0.046	129.1	0.38	0.957	0.956
HPP	8.719	-1.656	2.76	6.37	0.121				
Common model	8.649	-1.334	2.51	7.23	0.076	142.5	0.41	0.953	0.952

[a] k_1 in Eq. (6) was not statistically significant and in this case k_0 corresponds to the relative lag time (RLT).
[b] The parameter a corresponds to Log(N_{max}) at 0 °C.

From a practical point of view, it has been reported that HPP caused a reduction of the invasiveness of wild type *L. monocytogenes* isolates (Stollewerk et al., 2017) and piezo-tolerant mutants of *L. monocytogenes* seemed less virulent, and thus appear of lesser concern to human health than the wild type (Joerger et al., 2006; Karatzas et al., 2003). However, current detection and enumeration methods in food are not able to distinguish between these mutants and wild type cells. These issues are neither taken into account by the microbiological criteria regulations for *Listeria monocytogenes* nor by the guidelines to assess the safe shelf-life of RTE foods such as cooked ham (EURL Lm, 2014; European Commission, 2005). The assumption of equal growth potential of *L. monocytogenes* in both non-pressurised and pressurised meat products, stated in some risk assessments dealing with HPP products (Lerasle et al., 2014) is not supported by the results of the present study. Organic acids not only increase the growth rate of *L. monocytogenes* cells surviving HPP (Fig. 1), but they also protect the pathogen from the lethal effects of HPP (Bover-Cid et al., 2016). Consequently, the risk of non-compliance with microbiological criteria regulation could be higher than expected if these findings are not taken into account when designing and validating HPP for cooked meat products.

4 Conclusions

Besides a piezo-protective effect during processing, salts of organic acids exert a piezo-stimulating effect on surviving cells that can increase growth rate of *L. monocytogenes* in cooked ham as much as 4-fold. The mechanisms underlying this important piezo-stimulating effect remain to be elucidated. However, the present study emphasises the need of a product-oriented approach to design, evaluate and implement high pressure processing, taking into account the specific formulation used for product manufacture.

5 Acknowledgements

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Article 7

Enhancing high pressure bacterial inactivation by modified atmosphere packaging: effect of exposure time and cooked ham formulation

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Enhancing high pressure bacterial inactivation by modified atmosphere packaging: effect of exposure time and cooked ham formulation

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Abstract

High pressure processing (HPP) is a non-thermal preservation technology that can be applied as a control measure to inactivate pathogens and spoilage microorganisms once RTE meat products are packaged in a convenient format. HPP efficacy highly depends on product characteristics, but the impact of the sodium-reduced formulations and the effect of packaging atmosphere is scarcely known. The aim of the present work was to assess the effect of standard and sodium-reduced formulations from two different brands (A, B) under different packaging (vacuum and modified atmosphere packaging, MAP) on the HPP-inactivation kinetics of *Listeria monocytogenes* and spoilage lactic acid bacteria in cooked ham. Slices of cooked ham with standard and sodium-reduced formulations were inoculated with *L. monocytogenes* CTC1034 and *Latilactobacillus sakei* CTC746 (slime producer), packaged in vacuum and MAP (CO_2:N_2, 20:80) and pressurized (400MPa/0-15min) after 1h (vacuum, MAP) or 24h (MAP-exposed). Parameters of HPP-inactivation kinetics were estimated by fitting the Weibull model to log reduction data. Results showed that the efficacy of HPP in sodium-reduced cooked hams tended to decrease compared to standard formulations, being the difference statistically significant for *L. sakei*. The impact of MAP depended on the microorganism, the cooked ham brand and the exposure time before HPP. For *L. monocytogenes,* a significant enhancing effect of MAP was observed when HPP was applied just after packaging (1h, MAP) of cooked ham of brand A. In case of *L. sakei,* the inactivation by HPP was only enhanced in MAP-exposed samples. Therefore, the use of HPP as a control measure to inactivate relevant bacteria (either pathogenic or spoilage) must be applied through a product-oriented approach considering the type of packaging and the time-period between packaging and HPP.

Keywords:

High hydrostatic pressure; MAP; *Listeria monocytogenes*; Cooked meat products; foodborne pathogens; spoilage microorganisms.

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Article 8

Impact of packaging systems and high-pressure processing on the shelf-life of commercial cooked ham

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Abstract

Pre-packaged cooked ham is a perishable ready-to-eat meat product with a limited shelf-life due to its ability to support the growth of spoilage and pathogenic microorganisms. The "use by" date indicates the safe shelf-life of unopened packaged product but after opening the package (secondary shelf-life), storage conditions change and microbial growth usually increases. However, secondary shelf-life is often missed in quantitative microbial risk assessments (QMRA). The present work studied the growth of spoilage lactic acid bacteria and Listeria monocytogenes on cooked ham stored under different atmospheres with the aim was to determine the secondary self-life of pressurised (HPP) and non-HPP cooked ham with standard (ST) and sodium-reduced (SR) formulations.

L. monocytogenes CTC1034 and *Latilactobacillus sakei* CTC746 (slime producer) were inoculated on slices of commercial cooked ham and packaged in air, vacuum and MAP (20:80, CO_2:N_2). Half of the samples were pressurized at 600MPa/3min. All samples were stored at 6 °C for 3 months and *L. monocytogenes* and *L. sakei* were periodically enumerated on chromogenic and MRS agar, respectively. The logistic model was used to estimate the growth kinetic parameters and subsequently simulate the impact of opening the package after 4 days of storage.

Compared with ST formulation, in SR cooked ham growth rate of *L. monocytogenes* was higher, while that of *L. sakei* was lower. When lag time was considered, *L. sakei* reached the spoilage level (7 Log cfu/g) before *L. monocytogenes* reached the critical limit (100 cfu/g) in most of the products. However, the opposite was observed when no lag time for the pathogen was considered. The impact of atmosphere was only significant for *L. monocytogenes*, being its growth rate in air 30% and 43% higher than under vacuum and MAP, respectively. Consequently, when opening the package, the safe shelf-life (time to reach 100 cfu/g) was shortened up to 14% in VP and 47% in MAP cooked ham, both in HPP and non-HPP products. To avoid an overestimation of the food safety of cooked ham, QMRA should integrate the impact of secondary shelf-life, accounting for the reasonably foreseeable consumers habits regarding time and temperature conditions after opening the package.

Keywords: Date marking< High hydrostatic pressure, Time limit for consumption; Food Safety; Cooked meat products; *Listeria monocytogenes*; Spoilage.

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Article 9

A mathematical model to predict the antilisteria bioprotective effect of *Latilactobacillus sakei* CTC494 in vacuum packaged cooked ham

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A mathematical model to predict the antilisteria bioprotective effect of *Latilactobacillus sakei* CTC494 in vacuum packaged cooked ham

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Abstract

Biopreservation is a strategy that has been extensively covered by the scientific literature from a variety of perspectives. However, the development of quantitative modelling approaches has received little attention, despite the usefulness of these tools for the food industry to assess the performance and to set the optimal application conditions. The objective of this study was to evaluate and model the interaction between the antilisteria strain *Latilactobacillus sakei* CTC494 (sakacin K producer) and *Listeria monocytogenes* in vacuum-packaged sliced cooked ham. Cooked ham was sliced under aseptic conditions and inoculated with *L. monocytogenes* CTC1034 and/or *L. sakei* CTC494 in monoculture and coculture at 10:10, 10:10^3 and 10:10^5 cfu/g ratios of pathogen:bioprotective cultures. Samples were vacuum packaged and stored at isothermal temperature (2, 5, 10 and 15 °C). The growth of the two bacteria was monitored by plate counting. The Logistic growth model was applied to estimate the growth kinetic parameters (N_0, λ, μ_{max}, N_{max}). The effect of storage temperature was modelled using the hyperbola (λ) and Ratkowsky (μ_{max}) models. The simple Jameson-effect model, its modifications including the N_{cri} and the interaction γ factor, and the predator-prey Lotka Volterra model were used to characterize the interaction between both microorganisms. Two additional experiments at non-isothermal temperature conditions were also carried out to assess the predictive performance of the developed models through the Acceptable Simulation Zone (ASZ) approach. In monoculture conditions, *L. monocytogenes* and *L. sakei* CTC494 grew at all temperatures. In coculture conditions, *L. sakei* CTC494 had an inhibitory effect on *L. monocytogenes* by lowering the N_{max}, especially with increasing levels of *L. sakei* CTC494 and lowering the storage temperature. At the lowest temperature (2 °C) *L. sakei* CTC494 was able to completely inhibit the growth of *L. monocytogenes* when added at a concentration 3 and 5 Log higher than that of the pathogen. The inhibitory effect of the *L. sakei* CTC494 against *L. monocytogenes* was properly characterized and modelled using the modified Jameson-effect with interaction γ factor model. The developed interaction model was tested under non-isothermal conditions, resulting in ASZ values ≥ 83%. This study shows the potential of *L. sakei* CTC494 in the biopreservation of vacuum-packaged cooked ham against *L. monocytogenes*. The developed interaction model can be useful for the industry as a risk management tool to assess and set biopreservation strategies for the control of *L. monocytogenes* in cooked ham.

Keywords: *Listeria monocytogenes*, Lactic acid bacteria, microbial interaction models, bacteriocins, safe shelf-life, *Lactobacillus sakei*

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1 Introduction

Listeria monocytogenes is one of the relevant pathogens for ready-to-eat (RTE) cooked meat products due to the risk of listeriosis. Though it shows a relatively low morbidity, listeriosis severity is high, showing the highest hospitality and case-fatality rates among all foodborne bacteria, in the European Union (EU) accounting for 50% up to 67% of deaths depending on the year (ECDC & EFSA 2015; ECDC & EFSA 2019). Despite increasing awareness and the application of control measures focused on *L. monocytogenes* in food, listeriosis keeps a statistically significant increasing trend since 2009 (ECDC/EFSA, 2019). In 2019, the biggest listeriosis outbreak in EU occurred in Spain linked to the consumption of cooked meat products produced by a single manufacturer (WHO, 2019). Major listeriosis outbreaks occurring worldwide have also been linked to cooked meat products, e.g. Canada, 1985 (Maple Leaf Foods), USA (Farber et al., 2007), South Africa, 2017 (Thomas et al., 2020).

Food biopreservation consists of the use of microorganisms and/or their metabolites as an innocuous and ecological approach to extend the safe shelf-life of perishable products with minimal impact on the sensory characteristics. In the last decades, the interest towards a variety of microorganisms, particularly lactic acid bacteria (LAB), as bioprotective cultures to inhibit pathogenic bacteria, mainly *L. monocytogenes*, in meat products has been explored for long time by the scientific community (Amézquita & Brashears, 2002; Andersen, 1995; Bredholt et al., 1999; Bredholt et al., 2001; Budde et al., 2003; Danielski et al., 2020; Devlieghere et al., 2001; Hugas et al., 1998; Lucke, 2000; Mataragas et al., 2003; Rivas et al., 2014; Vermeiren et al., 2005). The psychotrophic nature of some LAB makes these bioprotective cultures a versatile strategy to control the growth of *L. monocytogenes* in foods, including chilled foods with extended shelf-life (Aymerich et al., 2006). Within LAB species, the use of bacteriocin producing strains with antilisteria activity can inhibit the growth and even compromise the viability of *L. monocytogenes* in meat products. Moreover, the production of bacteriocins in situ by the bioprotective cultures allow to avoid a possible loss of effectiveness of the bacteriocin when applicated (semi)purified in products, which in turn are not authorized as food preservatives in most of the countries (Aymerich et al., 2006). Instead, food cultures consisting of microbial species with Qualified Presumption of Safety (QPS) status according to the European Food Safety Authority (EFSA) or Generally Recognised As Safe (GRAS) to U.S. Food and Drug Administration can be used as food ingredients (Laulund et al., (2017). All these characteristics pointed out that LAB bioprotective cultures and their metabolites can be a feasible option to be considered by food manufacturers to extend the safe shelf-life of their perishable products, while complying with the regulated *L. monocytogenes* microbiological criteria applicable to RTE food stating a maximum acceptable limit of 100 cfu/g during the shelf-life of the food (e.g. Codex, 2007; European Commission, 2005; Health Canada, 2011).

Despite biopreservation through LAB has been extensively covered by the scientific literature from a variety of perspectives (e.g. potential technology applications, molecular mechanisms of action, etc.), the development of quantitative modelling approaches to address biopreservation has received much less attention so far. Predictive microbiology, also known as quantitative microbial ecology, is a useful approach to characterize and quantify the behaviour of microorganisms in food as a function of extrinsic and intrinsic factors through the use of primary and secondary models (Buchanan et al., 1997). The interaction between microbial groups (i.e. implicit factors) needs a bit more complex modelling approach (Cadavez et al., 2019). The competitive or antagonistic interactions between the bioprotective LAB culture and *L. monocytogenes* reported objective data on the antilisteria effect of the LAB cultures and on the consequent extension of the safe shelf-life. Within this context, interaction models that describe the simultaneous growth of each microorganism, taking into account the limited nutrient availability and the production of metabolites (lactic and acetic acids and bacteriocins among others) by LAB can be used to quantify the interaction of LAB and *L. monocytogenes* in real food matrices. Approaches based on the Jameson effect (Cornu et al., 2011) rely on the simultaneous deceleration of all microbial populations when the dominating microorganism reaches the stationary growth phase and inhibits the other to the same extend as they inhibit their own growth. In practice, this means that the maximum population density of *L. monocytogenes* is restricted by the growth of LAB. Jameson effect models were originally proposed to simulate the growth

of two populations in mix cultures based on growth parameters predicted from secondary growth models or estimated in pure cultures. In the case of incorporating, in the model, interaction-related parameters, the use of arbitrary values for these parameters may lead to discrepancies when predictions are compared with actual observed data (Cornu et al., 2011). Jameson effect models can also be used to fit growth curves with non-linear regression tools (Cornu et al., 2011), which provides adjusted values of the interaction related parameters leading to satisfactory predictive performance of the developed models (Costa et al., 2019). Jameson effect models are widespread and have been applied for describing microbial interaction between background microbiota and pathogens in milk, cheese, vegetables, fish and meat (Coleman et al., 2003; Giménez & Dalgaard, 2004; Guillier et al., 2008; Østergaard et al., 2014; Ross et al., 2000). Some modifications of this model have been proposed allowing to quantify the growth of the pathogen after the dominant (spoilage) microbial group reaches its maximum population (Giménez & Dalgaard, 2004) or to characterize the critical concentration of the dominant microorganism at which the pathogen stops growing (Le Marc et al., 2009). An empirical variant of the Jameson-effect model includes the use of the standard primary model for the population of interest and build a secondary model on the maximum population density parameter as a function of relevant environmental parameters (Cornu et al., 2011). A different approach is the one behind the Lotka-Volterra model, also known as a predator-prey model. Its underlying mechanism is the competition for a common substrate which allow to describe the dynamics of two interacting bacterial populations through competition factors that describe the reduction of the growth rate of a given population (Cornu et al., 2011; Powell et al., 2004; Valenti et al., 2013; Vereecken et al., 2000). Interaction models have been scarcely applied to quantitatively characterize the performance of bioprotective bacteria with specific antagonistic activities (e.g. through the bacteriocin production), which are intentionally added to the food at usually higher levels than those of the naturally present background (spoilage) microbiota.

In this framework, the objective of the present study was to quantitatively assess the effect of the sakacin-producing bioprotective strain *Latilactobacillus sakei* CTC494, previously *Lactobacillus sakei* (Zheng et al., 2020), on the inhibition of *L. monocytogenes* growth in vacuum packaged sliced cooked ham during refrigerated storage. This strain was previously reported to have antilisteria activity through the production of sakacin K (Hugas et al., 1993). The final purpose was to apply and validate a microbial interaction model to describe the interaction between both microorganisms at isothermal and non-isothermal conditions in order to provide food business operators with a versatile tool for the assessment and proper implementation of biopreservation for ensuring the safety of cooked ham during shelf-life.

2 Material and methods

2.1 Bacterial strains

The strain of *L. monocytogenes* used in the present study was the meat isolate CTC1034 (serotype 4b) from IRTA culture collection, previously used in our studies dealing with preservation of meat products (Bover-Cid et al., 2011, Bover-Cid et al., 2015; Bover-Cid et al., 2019; Hereu et al., 2012a; Hereu et al., 2012b; Hereu et al., 2014). As a bioprotective culture, the bacteriocin-producing *L. sakei* CTC494 strain was used. Sakacin K is the bacteriocin produced by this strain, which has been shown to inhibit the growth of spoilage bacteria and *Listeria* spp. (Hugas et al., 1993), including *L. monocytogenes* in different types of food (Aymerich et al., 2019; Costa et al., 2019; Hugas et al., 1995). The production of bacteriocin by the *L. sakei* CTC494 was confirmed the whole range of temperatures tested in the present study.

De Man Rogosa and Sharpe broth medium (MRS, Oxoid, UK) was used to store at -80 °C the *L. sakei* CTC494 strain stock culture while for the *L. monocytogenes* CTC1034 strain, the Brain Heart Infusion (BHI) broth (Beckon Dickinson, Sparks, Md., USA) was used. Both mediums were supplemented with 20% glycerol as cryoprotectant.

2.2 Cooked ham manufacture, sample preparation and inoculation

Cooked ham was manufactured using shoulder pork meat and the following ingredients (g/kg): water, 120; salt, 20.7; sodium tripolyphosphate, 5.8; dextrose, 5.8; carrageenan, 2.3; sodium ascorbate, 0.6; and sodium nitrite 0.1. Meat was minced in a cutter to a pArticle size of 6 mm. Ingredients were homogenized in a mixer for 30 min, stuffed into an impermeable plastic casing, and cooked in an oven at 68 °C for 5 h, the products reaching the core temperature of 65 °C. The manufactured cooked ham was composed of 1.60% fat, 19.56% protein, 75.64% moisture, 0.64% collagen, 2.72% salt (NaCl), 0.7% of lactic acid (endogenous) and with a pH 6.07 ± 0.03 and a_w 0.978 ± 0.001, in agreement with previous works (Bover-Cid et al., 2019). Cooked ham was sliced in the laboratory under aseptic conditions. Slices were spiked with either the *L. monocytogenes* CTC1034 strain or/and *L. sakei* CTC494 with 1% (v/w) of the corresponding culture diluted in physiological saline water (PSW, 0.85% w/v NaCl) to set up the required initial inoculum concentration. For monoculture experiments, both the pathogen and the bioprotective LAB were inoculated at ca. 10 cfu/g. For the coculture experiments, the pathogen concentration was also set up to ca. 10 cfu/g and for the bioprotective LAB three different initial concentrations were studied: 10, 10^3 and 10^5 cfu/g, respectively; making the concentration of bioprotective strain similar, 100-fold and 10000-fold higher than that of the pathogen, respectively. Therefore, the three initial concentration pathogen:bioprotective cultures studied corresponded to ratios 10:10, 10:10^3 and 10:10^5cfu/g. After inoculation, samples were vacuum packaged (EV-15-2-CD; Tecnotrip, Terrassa, Spain) in PET/PE bags (oxygen permeability < 50 cm^3/m^2/24 h and low water vapour permeability < 15 mg/m^2/24 h; Sacoliva S.L., Barcelona, Spain). Samples of each inoculation ratio were randomly distributed in four groups to be stored at 2, 5, 10 and 15 °C, respectively. The storage time ranged from 15 days (at the highest temperature) to 150 days (at the lowest temperature).

2.3 Monitoring bacterial concentrations along the storage time

Each sample (25 - 30 g) was homogenized 1/10 in a bag Blender Smasher® (bioMérieux, Marcy-l'Étoile, France) and 10-fold serially diluted in physiological saline solution (0.85% NaCl and 0.1% Bacto Peptone). Enumeration of *L. monocytogenes* was performed on the CHROMagar™ Listeria chromogenic media (CHROMagar, Paris, France) incubated at 37 °C for 48 h. To achieve a quantification limit of 2 cfu/g, 5 mL of the 1/10 diluted homogenate was pour plated into plates with a diameter of 14 cm (Hunt et al., 2017). For samples with expected concentration of *L. monocytogenes* below this quantification limit, the presence/absence of the pathogen was investigated by enrichment of 25 g-samples in 225 mL tryptic soy broth (Becton Dickinson) supplemented with 0.6% yeast extract (TSBYE) and incubated for 48 h at 37 °C. After enrichment, the presence of *L. monocytogenes* was detected by plating on CHROMagar™ Listeria. Enumeration of *L. sakei* CTC494 was conducted on de MRS agar media incubated at 30 °C for 72 h under anaerobiosis conditions. Endogenous LAB levels in cooked ham non-inoculated with bioprotective culture were below the limit of detection (10 cfu/g).

2.4 Primary growth modelling in monoculture conditions

The primary kinetic growth parameters of both *L. monocytogenes* CTC1034 and *L. sakei* CTC494 grown in monoculture conditions were estimated by fitting the Logistic growth models (Eq. 1, (Rosso et al., 1996)) without ($\lambda = 0$) and with delay ($\lambda > 0$) to the decimal logarithmic transformation of the respective observed counts. The need of the lag time (λ) was assessed with the F-test (Dalgaard, 1995). The *nls* and *nls2* functions from the respective *nls* and *nls2* R packages (R Core Team, 2019) were used in order to obtain the estimates of the primary kinetic parameters, their standard errors and the goodness of fit indicators (see section 2.8).

If t < λ; $\mathrm{Log}(N_t) = \mathrm{Log}(N_0)$

If t ≥ λ; $$\mathrm{Log}(N_t) = \mathrm{Log}\left(\frac{N_{max}}{1+\left(\left(\frac{N_{max}}{N_0}\right)+1\right)*\left(exp(-\mu_{max}*(t-\lambda))\right)}\right) \quad \text{Eq. (1)}$$

where *t* is time (days); N_0 is the bacterial concentration (cfu/g) at time zero; N_t is the bacterial concentration (cfu/g) at time t, N_{max} is the maximum population density (cfu/g), λ is the lag time (d) and μ_{max} is the maximum specific growth rate (d^{-1}).

2.5 Modelling the effect of L. sakei CTC494 on L. monocytogenes CTC1034 growth in coculture conditions by microbial interaction models

To study the interaction phenomenon between *L. monocytogenes* due to the growth of *L. sakei* CTC494, different interaction models were fitted to observed data in coculture experiments. With this approach, values of the interaction parameters could be properly estimated as suggested by Cornu et al. (2011), and then used as mathematical indicators of the nature of the microbial interaction.
The simultaneous growth of *L. monocytogenes* CTC1034 and *L. sakei* CTC494 during the storage of cooked ham at 2, 5, 10 and 15 °C was analysed through the fitting of the 4 microbial interaction models, i.e. the simple Jameson-effect model (Eq.2), two modified Jameson-effect models (with interaction *γ* factor and with N_{cri}, Eqs. 3 and 4, respectively) and the Lotka-Volterra model (Eq. 5) as shown in Table 1.

The Jameson-effect model was originally used to predict how the growth of all microbial populations (including pathogens) stops when the dominant microbial population (i.e. lactic acid bacteria, in this work *L. sakei* CTC494) reach its maximum population density *(N_{max}).* Under this Jameson effect, the other growth kinetic parameters of the pathogen, such as lag time and growth rate, remain unchanged by the dominant microbial population and thus can be determined from monoculture experiments (Cornu et al., 2011; Jameson, 1962). In the present work, interaction models based on the Jameson effect were used to fit the data and estimate the corresponding kinetic parameters under coculture conditions. In additions, the use of modifications in the Jameson-effect model were proposed to allow to estimate the behaviour of the pathogen (growth/no growth) after the lactic acid bacteria strain reaches the stationary phase. In this respect, the use of the interaction parameter *γ* allows the quantification of the inhibiting effect of the bioprotective lactic acid bacteria strain on *L. monocytogenes* growth as a function of temperature (Cadavez et al., 2019; Cornu et al., 2011; Giménez & Dalgaard, 2004; Møller et al., 2013). The estimation of the maximum critical concentration parameter (N_{cri}) refers to the level that *L. sakei* CTC494 should achieve to inhibit the growth of *L. monocytogenes* (Jameson, 1962; Le Marc et al., 2009; Vasilopoulos et al., 2010). Finally, the use of the simple Lotka-Volterra model (Cornu et al., 2011; Fujikawa et al., 2014; Giuffrida et al., 2008) allowed to estimate how the N_{max} of the bioprotective *L. sakei* CTC494 affected the growth of the *L. monocytogenes* CTC1034 through the competition factor. Depending on the value of the competition factor parameters of *L. sakei* CTC494 on *L. monocytogenes* CTC1034 of the Lotka-Volterra model (F_{LsLm} and F_{LmLs}), *L. monocytogenes* could stop growing (F_{LsLm} =1), grow with reduced μ_{max} (0 < F_{LsLm} < 1) or decline population when *L. sakei* reached its N_{max} (F_{LsLm} >1).

Ordinary differential equations (ODE) included in the interaction models were solved analytically using the numerical Runge-Kutta method (Butcher, 2003) and minimizing the residual sum of squares (RSS) of the errors throughout the search of the most suitable parameter of the interaction model. Parameter estimation by least-square optimization was performed with the "*deSolve*" and "*FME*" packages implemented in the R software (R Core Team, 2019; Cornu et al., 2011).

Table 1. Interaction models evaluated in the present study.

Interaction model	Formula	
Simple Jameson-effect	$t < \lambda_{Ls}$,	$\frac{dN_{Ls}}{dt} = 0$
	$t \geq \lambda_{Ls}$,	$\frac{dN_{Ls}}{dt} = N_{Ls} \cdot \mu_{maxLs} \cdot \left(1 - \frac{N_{Ls}}{N_{maxLs}}\right) \cdot \left(1 - \frac{N_{Lm}}{N_{maxLm}}\right)$
	$t < \lambda_{Lm}$,	$\frac{dN_{Lm}}{dt} = 0$
	$t \geq \lambda_{Lm}$,	$\frac{dN_{Lm}}{dt} = N_{Lm} \cdot \mu_{maxLm} \cdot \left(1 - \frac{N_{Lm}}{N_{maxLm}}\right) \cdot \left(1 - \frac{N_{Ls}}{N_{maxLs}}\right)$
Modified Jameson-effect with γ	$t < \lambda_{Ls}$,	$\frac{dN_{Ls}}{dt} = 0$
	$t \geq \lambda_{Ls}$,	$\frac{dN_{Ls}}{dt} = N_{Ls} \cdot \mu_{maxLs} \cdot \left(1 - \frac{N_{Ls}}{N_{maxLs}}\right) \cdot \left(1 - \frac{N_{Lm}}{N_{maxLm}}\right)$
	$t < \lambda_{Lm}$,	$\frac{dN_{Lm}}{dt} = 0$
	$t \geq \lambda_{Lm}$,	$\frac{dN_{Lm}}{dt} = N_{Lm} \cdot \mu_{maxLm} \cdot \left(1 - \frac{N_{Lm}}{N_{maxLm}}\right) \cdot \left(1 - \frac{\gamma \cdot N_{Ls}}{N_{maxLs}}\right)$
Modified Jameson-effect with N_{cri}	$t < \lambda_{Ls}$,	$\frac{dN_{Ls}}{dt} = 0$
	$t \geq \lambda_{Ls}$,	$\frac{dN_{Ls}}{dt} = N_{Ls} \cdot \mu_{maxLs} \cdot \left(1 - \frac{N_{Ls}}{N_{maxLs}}\right) \cdot \left(1 - \frac{N_{Lm}}{N_{criLm}}\right)$
	$t < \lambda_{Lm}$,	$\frac{dN_{Lm}}{dt} = 0$
	$t \geq \lambda_{Lm}$,	$\frac{dN_{Lm}}{dt} = N_{Lm} \cdot \mu_{maxLm} \cdot \left(1 - \frac{N_{Lm}}{N_{maxLm}}\right) \cdot \left(1 - \frac{N_{Ls}}{N_{criLs}}\right)$
Simplified Lotka-Volterra	$t < \lambda_{Ls}$,	$\frac{dN_{Ls}}{dt} = 0$
	$t \geq \lambda_{Ls}$,	$\frac{dN_{Ls}}{dt} = N_{Ls} \cdot \mu_{maxLs} \cdot \left(1 - \frac{N_{Ls} + F_{LsLm} \cdot N_{Lm}}{N_{maxLs}}\right)$
	$t < \lambda_{Lm}$,	$\frac{dN_{Lm}}{dt} = 0$
	$t \geq \lambda_{Lm}$,	$\frac{dN_{Lm}}{dt} = N_{Lm} \cdot \mu_{maxLm} \cdot \left(1 - \frac{N_{Lm} + F_{LmLs} \cdot N_{Ls}}{N_{maxLm}}\right)$

Eq. (2)

Eq. (3)

Eq. (4)

Eq. (5)

where for *L. sakei* CTC494 (*Ls*) and *L. monocytogenes* (*Lm*), λ is the lag time (d), N is the bacterial concentration (Log cfu/g) at time t, μ_{max} is the maximum specific growth rate (d^{-1}), N_{max} is the maximum population density (Log cfu/g), γ is a interaction factor that allows *L. monocytogenes* to increase (γ <1) or decrease (γ >1) after *L. sakei* has reached its N_{max}, N_{cri} is the maximum critical concentration that a population should reach to inhibit the growth of the other population, F_{LsLm} and F_{LmLs} are the competition factors of one species on the other.

2.6 Secondary modelling

Secondary modelling was applied to assess the effect of the storage temperature on the primary growth parameters of *L. monocytogenes* CTC1034 and *L. sakei* CTC494 in cooked ham obtained through the fitting of the Logistic growth models to monoculture data and with the fitting of the interaction models to coculture data. For coculture conditions, the impact of the *L. sakei* CTC494 inoculum level on the primary growth parameters of both microorganisms was also assessed.
The hyperbola model (Zwietering et al., 1994) was used to fit the lag time (λ) values determined at different temperatures.

$$\lambda = {a_1}/{(T - b_1)} \quad \text{Eq. (6)}$$

where a_1 and b_1 are constant parameters and T is the storage temperature (°C)
The square root model (Eq. 7) (Ratkowsky et al., 1982) was used to estimate the effect of the storage temperature on the growth rate (μ_{max}) of *L. monocytogenes* CTC1034 and *L. sakei* CTC494 obtained in the primary modelling.

$$\sqrt{\mu_{max}} = a_2 \cdot (T - b_2) \quad \text{Eq. (7)}$$

where a_2 is a constant parameter, T (°C) is the storage temperature and b_2 is T_{min} (°C) corresponding to the theoretical minimum growth temperature for each microorganism.
The effect of storage temperature on the maximum population density of the microorganism (N_{max}) was described by using a second degree polynomial equation as in Eq. 8.

$$\mathrm{Log}\, N_{max} = a_3 \cdot T^2 + b_3 \cdot T + c \quad \text{Eq. (8)}$$

where a_3 and b_3 are slope parameters and c corresponds to N_{max} at 0 °C.

The fit of the secondary models was conducted with *nls* and *nls2* functions from the respective *nls* and *nls2* packages included in the R software (R Core Team, 2019).

2.7 Goodness of fit and predictive model performance

Parameter estimates from the models were evaluated with the standard error. Moreover, for the interaction models, the significance of the parameter (*p*-value) was recorded. For all models, the goodness of fit was assessed in terms of RMSE. The adjusted coefficient of determination (R^2_{adj}) was also used to assess the goodness of fit of secondary the linear models (i.e. Eq 7 and Eq8).
To assess the predictive performance of the interaction models for *L. sakei* CTC494 and *L. monocytogenes* CTC1034, an independent experiment was carried out. The two microorganisms were inoculated in the same type of cooked ham and ratios (10:10, $10:10^3$ and $10:10^5$ cfu/g), vacuum packaged as described above and exposed to 2 non-isothermal profiles, one with mean temperature 2.99 °C and range from 2.4 °C to 9.1 °C (profile 1) and another with mean temperature 3.62 °C ranging between 0 °C and 20 °C (profile 2). The growth of the bioprotective culture and the pathogen was monitored as described in section 2.3. To simulate simultaneous growth of both microorganisms in the two non-isothermal profiles, interaction growth models were applied using appropriate secondary models and specific values of the kinetic parameters (N_{max}) obtained in monoculture and interaction parameter (γ) values derived from the experiments under coculture conditions at constant temperatures. The predictive performance was evaluated with the acceptable simulation zone (ASZ) approach. Model simulation was considered acceptable when at least 70% of the predictions were within the ASZ. In this case, the intrinsic variability of

the *L. monocytogenes* data when challenged with *L. sakei* CTC494 (in some cases higher than 1 Log) implied to define the ASZ as the difference of ± 1 Log unit between the observed and predicted bacterial concentration by the developed model as suggested by Møller et al.(2016).

3 Results and discussion

3.1 Growth of L. sakei CTC494 and L. monocytogenes CTC1034 in monoculture conditions

3.1.1 Primary modelling

Growth of *L. sakei* CTC494 and *L. monocytogenes* CTC1034 in monoculture are shown in Figure 1 (a-d and e-h, respectively) with the estimated kinetic parameters obtained from the fitting of the Logistic growth models without and with delay to Log count data reported in Table 2. No significant lag time (λ) was observed for the bioprotective *L. sakei* CTC494, indicating that this strain was well adapted. On the contrary, *L. monocytogenes* CTC1034 required a time for adaptation before starting to grow and the λ increased with decreasing the storage temperature, being statistically significant for all conditions assayed. Moreover, λ of *L. monocytogenes* was influenced by the *L. sakei* CTC494 initial concentrations, as also reported by Quinto et al. (2016) and Mejlholm & Dalgaard (2015).

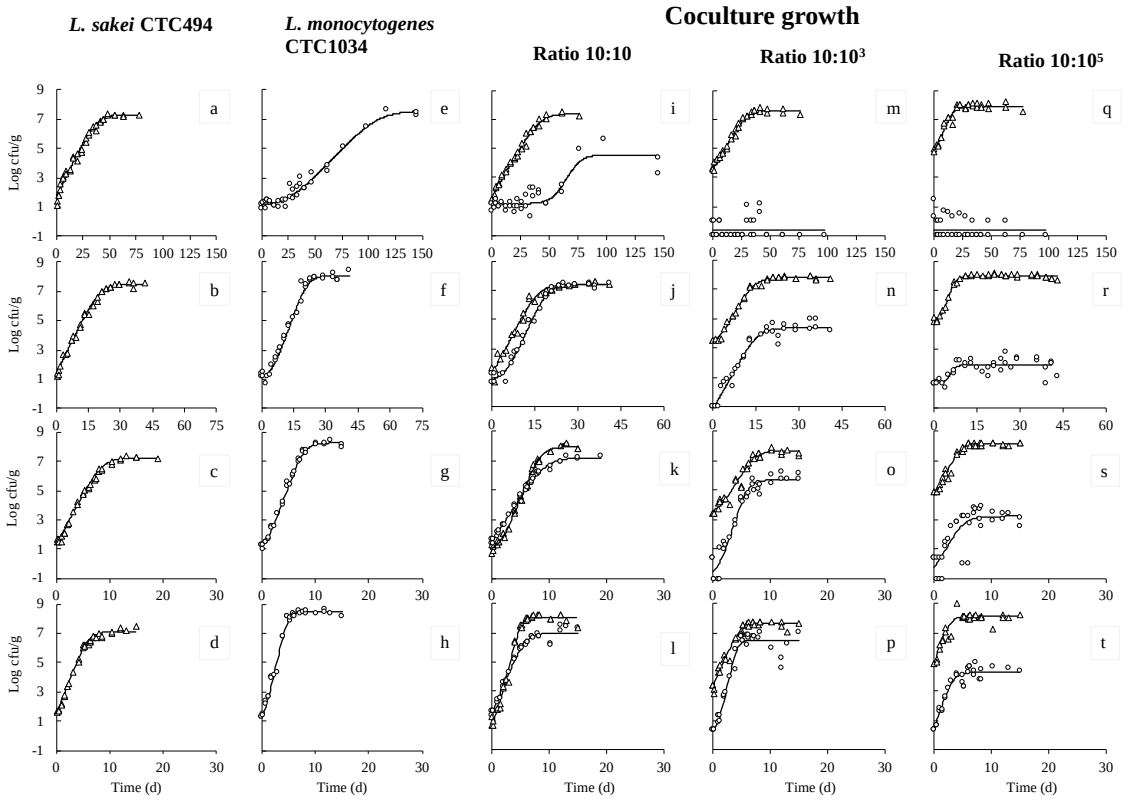

Figure 1. Observed counts for *L. sakei* CTC494 (triangles) and *L. monocytogenes* CTC1034 (circles) in cooked ham stored at 2, 5, 10 and 15 °C in monoculture and coculture conditions. Lines represent the fit of the Logistic growth model without and with delay for the *L. sakei* CTC494 and *L. monocytogenes* CTC1034 growth, respectively, in monoculture conditions and the fit of the Jameson-effect with interaction γ model on the growth of *L. sakei* CTC1034 and *L. monocytogenes* CTC1034 in coculture conditions.

For both microorganisms, higher growth rates (μ_{max}) were obtained with increasing the storage temperature. At higher temperatures (10 and 15 °C), higher μ_{max} were found for *L. monocytogenes* CTC1034 compared to those observed for *L. sakei* CTC494, indicating that *L. monocytogenes* presented a better ability to grow in cooked ham stored at abusive storage temperatures when grown in monoculture.

The opposite happened at the lowest temperature tested, where higher μ_{max} was found for *L. sakei* CTC494 compared to the pathogen.

Table 2. Estimated parameter values resulting from the fit of the Logistic growth model without and with delay (Eq. 1) to the *L. sakei* CTC494 and *L. monocytogenes* CTC1034 counts, respectively, in monoculture conditions in cooked ham during storage at 2, 5, 10 and 15 °C.

Microorganism	Temperature (°C)	Kinetic parameters[a]				Goodness of fit[b]	
		Log N_0 (Log cfu/g)	λ (days)	μ_{max} (Ln/d)	Log N_{max} (Log cfu/g)	n	RMSE
L. sakei CTC494	2	1.76 ± 0.09	-	0.32 ± 0.01	7.21 ± 0.09	37	0.269
	5	1.45 ± 0.08	-	0.64 ± 0.02	7.42 ± 0.07	34	0.218
	10	1.44 ± 0.06	-	1.47 ± 0.03	7.19 ± 0.07	35	0.186
	15	1.53 ± 0.08	-	2.01 ± 0.06	7.02 ± 0.07	34	0.223
L. monocytogenes CTC1034	2	1.24 ± 0.08	21.8 ± 2.6	0.16 ± 0.01	7.50 ± 0.21	37	0.319
	5	1.18 ± 0.11	3.7 ± 0.6	0.84 ± 0.03	8.00 ± 0.09	33	0.275
	10	1.22 ± 0.09	0.7 ± 0.2	2.14 ± 0.05	8.19 ± 0.07	34	0.180
	15	1.39 ± 0.12	0.4 ± 0.1	3.41 ± 0.13	8.38 ± 0.06	33	0.234

[a] Parameter estimate ± standard error. Log N_0 is the initial bacterial concentration (Log cfu/g), λ is the lag time (d), μ_{max} is the maximum specific growth rate (d^{-1}), Log N_{max} is the maximum population density (Log cfu/g).
[b] n: number of data points, RMSE: root mean squared error.

Besides the effect on the μ_{max}, the storage temperature did not significantly affect the maximum population density (N_{max}) of the studied bacteria, with an average close to 7 Log cfu/g for *L. sakei* CTC494 and 8 Log cfu/g for *L. monocytogenes* CTC1034. Therefore, when grown in monoculture without interaction, the pathogen generally grew faster and achieved a higher population density than the bioprotective culture, except at 2°C in which *L. monocytogenes* grew slower that the *L. sakei* CTC494.

3.1.2 Secondary modelling

As *L. sakei* CTC494 started immediately to grow in all conditions, the effect of the storage temperature on the λ was only studied for the *L. monocytogenes* strain. The increase of λ with decreasing the storage temperatures was non-linear and it could be properly quantified throughout the fit of the hyperbola model to the estimated λ (Eq. 6, Table 4).

The square root model (Eq. 7, Table 4) was used to quantify the effect of the storage temperature on the μ_{max}. Though *L. sakei* CTC494 grew faster than *L. monocytogenes* at lower temperatures, the bioprotective LAB was less sensitive to temperature changes as shown in Figure 2, i.e., the slope of the secondary model was steeper for the pathogen and thus at the higher temperatures assessed the growth rate of the pathogen was above that of *L. sakei* CTC494. It is worth to highlight that the growth rates observed for the bioprotective strain *L. sakei* CTC494 were notably lower than the growth predicted for spoilage *Lactobacillus* (not specifically producing bacteriocins) by the model "Growth of *Lactobacillus* in seafood and meat products" included in the Food Spoilage and Safety Predictor (FSSP v4.0) (Mejlholm & Dalgaard, 2007; Mejlholm et al., 2015; Mejlholm & Dalgaard, 2015) considering the same physicochemical characteristics as those of the cooked ham of the present study (pH of 6.07, 2.71% water phase salt and 7034 ppm endogenous lactic acid) (Figure 2). On the contrary, the meat *L. monocytogenes* CTC1034 strain used in the present work showed higher growth capability than the predicted by the "Growth of *Listeria monocytogenes* in chilled seafood and meat products" model of the FSSP. This finding is also in agreement with the previous work (Serra-Castelló et al., 2018) where the *L. monocytogenes* CTC1034 was also shown to grow faster in vacuum packaged cooked ham than the strain 12MOB089LM recommended by the EU Reference laboratory for conducting challenge test studies in refrigerated meat products (EURL Lm, 2014). Thus, considering all these results, the present work would cover a worse-case scenario of a fast-growing *L. monocytogenes*.

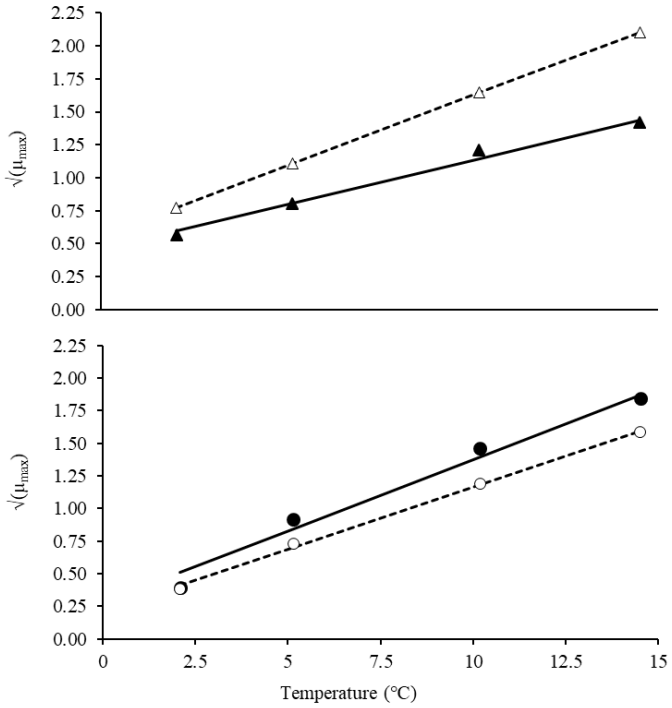

Figure 2. Fit of the square root model (lines) to the growth rates (μ_{max}) found for *L. sakei* CTC494 (triangles, top plot) and *L. monocytogenes* (circles, bottom plot) in monoculture conditions. Closed symbols and continuous lines represent the observed μ_{max} values and the corresponding model fit. Open symbols and dashed lines represent the predictions for lactic acid bacteria and *L. monocytogenes* provided by the FSSP model.

Regarding N_{max}, the lack of fit obtained when fitting the polynomial model (Eq. 8) to N_{max} parameters (data not shown) corroborated that storage temperature did not significantly affect the N_{max} parameter of either *L. sakei* CTC494 and *L. monocytogenes* CTC1034. Therefore, the mean of the estimated N_{max} obtained for all temperatures was assumed to be representative.

3.2 Behaviour of L. sakei CTC494 and L. monocytogenes CTC1034 in coculture conditions

Growth of *L. monocytogenes* CTC1034 in the presence of different initial concentrations of the bioprotective *L. sakei* CTC494 and stored at the different temperatures is shown in Figure 1 (i-t).
Results of the present study showed that *L. monocytogenes* was able to growth in most of the conditions although to a much lower extent than that observed in monoculture conditions and with a higher variability within the observed counts (slightly wider dispersion of data within a growth curve). Microbiological analysis of the cooked ham revealed that endogenous LAB was below 10 cfu/g indicating that the behaviour of *L. monocytogenes* in coculture conditions was mainly conditioned by *L. sakei* CTC494 and not by endogenous bacteria. The main impact of the *L. sakei* CTC494 strain was the decrease of the maximum population density of the pathogen, which is a very relevant parameter determining the risk of listeriosis according to the quantitative microbial risk assessments (QMRA) developed so far (Pérez-Rodríguez et al., 2017).

Table 3. Estimated parameter values resulting from the fit of the Jameson-effect model with interaction gamma (γ) factor (Eq. 3) model to the *L. sakei* CTC494 and *L. monocytogenes* CTC1034 counts in coculture conditions in cooked ham during storage at 2, 5, 10 and 15 ˚C.

Temperature (˚C)	Ratio	Kinetic parameters[a]								Goodness of fit[b]	
		λ_{-Lm} (d)	$N_{0\text{-}Ls}$ (Log cfu/g)	$N_{0\text{-}Lm}$ (Log cfu/g)	$\mu_{max\text{-}Ls}$ (Ln/d)	$\mu_{max\text{-}Lm}$ (Ln/d)	$N_{max\text{-}Ls}$ (Log cfu/g)	$N_{max\text{-}Lm}$ (Log cfu/g)	γ	n	RMSE
2	10:10	56.0 ± 10.0	1.63 ± 0.06	1.09 ± 0.08	0.31 ± 0.01	0.20 ± 0.02	7.34 ± 0.06	4.75 ± 0.08	0.19 ± 0.04	38	1.000
2	10:10^3	-	3.53 ± 0.14	-0.57 ± 0.11	0.41 ± 0.06	0.60 ± 2.12	7.51 ± 0.15	0.03 ± 0.21	1.07 ± 0.22	34	1.012
2	10:10^5	-	4.77 ± 0.17	-0.45 ± 0.09	0.46 ± 0.09	0.19 ± 0.49	7.86 ± 0.12	0.33 ± 0.24	1.17 ± 0.43	34	0.876
5	10:10	2.0 ± 0.7 [c]	1.62 ± 0.10	1.09 ± 0.10	0.74 ± 0.02	1.02 ± 0.04	7.51 ± 0.22	7.37 ± 0.60	1.00[d]	42	0.685
5	10:10^3	2.8 ± 1.1 [c]	3.72 ± 0.06	-0.96 ± 0.05	0.76 ± 0.04	0.83 ± 0.03	7.86 ± 0.06	4.34 ± 0.06	1.00[d]	40	0.669
5	10:10^5	4.2 ± 2.3 [c]	5.21 ± 0.08	0.82 ± 0.11	1.69 ± 0.13	1.48 ± 0.21	7.98 ± 0.08	2.15 ± 0.15	1.00[d]	46	0.692
10	10:10	1.3 ± 0.2 [c]	1.40 ± 0.09	1.32 ± 0.04	1.42 ± 0.05	2.10 ± 0.06	7.01 ± 0.03	8.18 ± 0.05	1.00[d]	40	0.539
10	10:10^3	1.2 ± 0.4 [c]	3.78 ± 0.06	-0.20 ± 0.05	1.72 ± 0.13	2.43 ± 0.13	7.55 ± 0.12	5.87 ± 0.11	1.00[d]	40	0.938
10	10:10^5	1.4 ± 0.6 [c]	5.03 ± 0.07	-0.18 ± 0.07	2.06 ± 0.11	2.22 ± 0.12	8.09 ± 0.06	3.75 ± 0.12	1.00[d]	40	0.758
15	10:10	0.5 ± 0.1 [c]	1.28 ± 0.18	0.37 ± 0.19	2.39 ± 0.14	3.31 ± 1.44	6.87 ± 0.12	8.22 ± 0.42	1.00[d]	36	0.765
15	10:10^3	0.7 ± 0.2 [c]	3.14 ± 0.09	0.66 ± 0.09	2.14 ± 0.13	3.63 ± 0.22	8.21 ± 0.09	6.49 ± 0.07	1.00[d]	38	1.117
15	10:10^5	0.0 ± 0.5 [c]	4.99 ± 0.22	0.38 ± 0.14	2.08 ± 0.23	2.66 ± 0.20	8.22 ± 0.26	4.34 ± 0.11	1.00[d]	38	0.976

[a] Parameter estimate ± standard error. Where for *L. sakei* CTC494 (*Ls*) and *L. monocytogenes* (*Lm*), N_0 is the initial bacterial concentration (Log cfu/g), μ_{max} is the maximum specific growth rate (d^{-1}), γ is a interaction factor that allows *L. monocytogenes* to increase (γ <1) or decrease (γ >1) after *L. sakei* has reached its N_{max} and N_{max} is the maximum population density (Log cfu/g).

[b] n: number of data points, RMSE: root mean squared error.

[c] Parameter no significant.

[d] γ parameter was fixed to a value of 1

At higher initial *L. monocytogenes*:*L. sakei* ratio and at lower storage temperatures, the growth of *L. monocytogenes* was more inhibited than at lower ratios and at higher temperatures pointing out that the initial concentration of the dominant microorganism (the bioprotective *L. sakei* strain) and temperature are key factors determining the level of inhibition of the pathogen. This has also been described for the interaction between the pathogen and spoilage LAB (Mellefont et al., 2008). It is worth to highlight the behaviour of *L. monocytogenes* at 2 °C in the presence of *L. sakei* CTC494. At the ratio 10:10 (i.e. equal low concentration for both the pathogen and the bioprotective culture) the lag phase of *L. monocytogenes* was almost tripled in comparison with the monoculture growth (i.e. from 21.8 days to 56 days), and no growth of *L. monocytogenes* was recorded during the first 8 weeks of storage. The impact of the bioprotective culture when the initial *L. sakei* CTC494 concentrations were higher than the pathogen (ratios $10:10^3$ and $10:10^5$) was much greater, resulting in the total lack of growth of *L. monocytogenes,* with a slight tendency to die-off during the chill storage. These results suggested that the lower temperature together with the higher initial *L. sakei* concentration exerted additive effects on the *L. monocytogenes* growth inhibition, compromising its viability in the most unfavourable growth conditions. The antilisteria effect can be mainly attributed to the production of specific metabolites with listeria-inhibitory effect (i.e. sakacin K, De Vuyst & Leroy, 2007; Hugas et al., 1995; Leroy et al., 2005; Ravyts et al., 2008), though the eventual exhaustion of critical nutrients may also play a role. Since *L. sakei* CTC494 shows to be a low acidifying strain (Hugas et al., 1995), no relevant changes in the pH of cooked ham were recorded during the storage time (data not shown), thus the influence of other metabolites such as organic acids on the *L. monocytogenes* growth was considered to be much less relevant than sakacin K.

Overall, the above results showed that the bioprotective strain *L. sakei* CTC494 can be used as a food biopreservation strategy for the control of *L. monocytogenes* growth extending thus, the safe shelf-life of cooked ham with minimal impact on the sensory characteristics, as no slime nor gas or off-odours were detected (data not shown), in agreement with (Aymerich et al., 2002). The bioprotective potential of the *L. strain* CTC494 has also been shown for other meat products such as fermented sausages (acting as a starter culture (Hugas et al., 1995; Leroy & De Vuyst, 2003), showing a significant listericidal effect as it coupled with the acid production and the decrease of a_w due to sausage drying) and for fish (Aymerich et al., 2019; Costa et al., 2019).

3.3 Modelling the bioprotective effect of L. sakei CTC494 on L. monocytogenes CTC1034 growth by microbial interaction models

For storage temperatures above 5 °C, results of the coculture conditions showed that the inhibition of the *L. monocytogenes* growth occurred when the dominant population, i.e., *L. sakei* CTC494 reached their N_{max} (Figure 1k-t). These results would indicate that the Jameson-effect model could properly fit the simultaneous growth of the bioprotective LAB culture and the pathogen data. However, at the lowest storage temperature studied (2 °C) the *L. monocytogenes* behaviour was strongly conditioned by the level of the bioprotective culture (Figure 1i, 1m and 1q). For the ratio 10:10, the pathogen continued growing after *L. sakei* CTC494 reached its N_{max}; while at higher ratios (ratios $10:10^3$ and $10:10^5$), *L. monocytogenes* was unable to grow, showing even a slight die off trend during the storage period. These results suggested the need of models (Table 1, Eq. 3, Eq. 4 and Eq. 5) that shed light on the *L. monocytogenes* behaviour after *L. sakei* CTC494 achieved its N_{max}. Estimated parameters from the different models and their goodness of fit for simultaneous growth of *L. sakei* CTC494 and *L. monocytogenes* CTC1034 in cooked ham stored at 2, 5, 10 and 15 °C are reported in the Supplementary Table 1. The 3 variants of the Jameson-effect model appeared statistically adequate to quantitatively describe the simultaneous growth of *L. sakei* and *L. monocytogenes* in cooked ham at all studied storage temperatures, since most of the estimated parameters were significant (p-value <0.05). On the contrary, most of the estimated parameters of the Lotka-Volterra model were not significant (p-value >0.05), especially those defining the interaction between both microorganisms (F_{LsLm}, F_{LmLs}). Therefore, according to these data, Lotka-Volterra model was

not the most appropriate interaction model to fit the simultaneous growth of *L. sakei* and *L. monocytogenes* in cooked ham.

Regarding the Jameson-effect models, the common estimated parameters from the 3 interaction models (N_0, μ_{max} and N_{max}) were very close and in most of the conditions, models yielded a good fit (low RMSE). Although that, statistically significant differences in the goodness of fit were found when fitting models to *L. sakei* and *L. monocytogenes* data at 2 °C, being the modified Jameson-effect models with interaction γ factor and with N_{cri} the models that gave the best fit. Both models reported valuable information about the effect of *L. sakei* CTC494 on *L. monocytogenes* growth. The fit of the Jameson-effect model with N_{cri} indicated that while at higher temperatures (10 and 15 °C) the critical *L. sakei* CTC494 level at which *L. monocytogenes* stopped growing tended to be lower than the N_{max} of *L. sakei* CTC494, the opposite was predicted at 5 °C. Thus, these results suggested that at higher temperatures, the bioprotective effect of *L. sakei* CTC494 was the main cause of the decrease of the N_{max} values of *L. monocytogenes*. On the other hand, the Jameson-effect model with interaction γ factor, for temperatures ≥ 5°C, γ parameter was equal to 1 as *L. monocytogenes* stopped growing when *L. sakei* achieved its N_{max}, leading to a simple Jameson-effect model. At 2 °C, the fit of the model resulted in γ values of 0.19, 1.07 and 1.17 (Table 3) for pathogen:bioprotective strain ratios of 10:10, $10:10^3$ and $10:10^5$, respectively, allowing to properly quantify the growth (γ <1) and the inactivation (γ >1) of *L. monocytogenes* after *L. sakei* CTC494 achieved its N_{max}. Overall, these results agree with those of Costa et al. (2019) dealing with raw fish, which suggest that the interaction between the bioprotective strain and the pathogen could be explained by a combination of two main mechanisms. On the one hand, by the non-specific interaction as considered by the classical Jameson effect; in the present study this was evident for the experiments with initial concentration of *L. sakei* CTC494 higher than that of the pathogen enabling the bioprotective strain to reach the maximum population density before *L. monocytogenes* (Cornu et al., 2011; Mellefont et al., 2008; Jameson, 1962). On the other hand, pathogen inhibition was further enhanced by the specific interaction probably caused by the antagonistic effect of the bacteriocin produced (i.e. sakacin K) by the bioprotective strain (Aguilar and Klotz, 2010; Costa et al., 2019; Vescovo et al., 2006). Moreover, the results showed that this specific interaction was affected by the storage temperature, especially at lower temperature values (< 5 °C), where *L. monocytogenes* maximum population was not only affected by the initial *L. sakei* concentration but also by the storage temperature. Therefore, the modified Jameson-effect model with interaction γ factor that allows to quantify the inhibiting effect of *L. sakei* CTC494 on the *L. monocytogenes* CTC1034 growth as a function of temperature was chosen for properly characterize the behaviour (either growth and/or slight inactivation) of the pathogen observed at low temperature (2 °C).

Regarding parameter estimation, results from the fitting of the modified Jameson-effect model with interaction γ factor showed that the growth rates found for *L. sakei* CTC494 and *L. monocytogenes* CTC1034 in coculture conditions (Table 3) were not significantly ($p>0.05$) different from those found in monoculture conditions (Table 2) at the same storage temperature. Therefore, the growth rate of *L. monocytogenes* was not affected by the initial concentrations of *L. sakei* CTC494 studied in the present study. These results were in agreement with those reported by the model included in the FSSP application ("Growth of *L. monocytogenes* and *LAB* in chilled seafood and meat products" model) (Mejlholm & Dalgaard, 2007; Mejlholm & Dalgaard, 2015; Mejlholm et al., 2015) where the growth rate of *L. monocytogenes* strains was not affected by the concentration of the *Lactobacillus* strains. On the contrary, the *L. monocytogenes* of N_{max} was significantly reduced with increasing the ratio of *L. sakei*. N_{max} estimates of *L. monocytogenes* were reduced by 2 Log with increasing 100-fold the *L. sakei* initial concentration, emphasizing the greatest effect of *L. sakei* on the N_{max} of *L. monocytogenes*. Therefore, the results indicated that *L. sakei* influenced the growth pattern of *L. monocytogenes* mainly by reducing the maximum population density of the pathogen. Thus, *L. sakei* CTC494 emerged as a feasible bioprotective strategy to control and reduce the *L. monocytogenes* concentration during the cooked ham safe shelf-life in conditions where *L. monocytogenes* could grow.

3.4 Quantifying the effect of the storage temperature and L. sakei CTC494 initial concentration on the primary kinetic parameters of L. monocytogenes

To further characterize and quantify the impact of the storage temperature and *L. sakei* CTC494 initial concentration on the kinetic parameters obtained from the interaction models, the secondary modelling approach was applied. Same secondary models used in monoculture conditions were used to quantify the impact of storage temperature and initial *L. sakei* CTC494 levels on the kinetic parameters obtained from Jameson-effect model with interaction *γ* factor (N_0, μ_{max}, N_{max} and *γ*) describing the simultaneous growth of the bioprotective strain and the pathogen.

Table 4. Fit of the secondary models to the primary kinetic parameters obtained with the Jameson-effect with interaction *γ* model.

Experimental conditions	Secondary model	Microorganism		Parameters[a]			Goodness of fit[b]			
			Ratio	a_n	b_n	c	n	p	RMSE	R^2_{adj}
Monoculture	$\lambda = {}^{a_1}/_{(T-b_1)}$	*L. monocytogenes*	-	11.42 ± 2.22	1.48 ± 0.10	-	4	2	0.604	0.996
	$\sqrt{\mu_{max}} = a_2 \cdot (T - b_2)$	*L. sakei* CTC494	-	0.07 ± 0.05	-6.96 ± 10.62	-	4	2	0.070	0.967
		L. monocytogenes	-	0.12 ± 0.02	-2.56 ± 1.64	-	4	2	0.127	0.960
Coculture	$\sqrt{\mu_{max}} = a_2 \cdot (T - b_2)$	*L. sakei* CTC494	-	0.06 ± 0.03	-9.45 ± 9.70	-	4	2	0.161	0.888
		L. monocytogenes	-	0.09 ± 0.07	-6.09 ± 13.03	-	10	2	0.163	0.958
	$N_{max} = a_3 \cdot T^2 + b_3 \cdot T + c$	*L. monocytogenes*	10:10	-0.04 ± 0.02	0.93 ± 0.30	3.27± 1.02	4	3	1.430	0.856
			$10:10^3$	-0.06 ± 0.03	1.48 ± 0.52	-2.31 ± 1.74	4	3	2.417	0.870
			$10:10^5$	-0.03 ± 0.00	0.74 ± 0.07	-1.00 ± 0.21	4	3	0.297	0.995

[a] Parameter estimate ± standard error. For growth rate the b_2 parameter corresponds to T_{min} (see Eq.7)
[b] n: number of data points, p: number of parameters estimated, RMSE: root mean squared error and R^2_{adj}: adjusted coefficient of determination, R^2_{adj}: adjusted coefficient of determination.

Challenging microorganisms in coculture conditions did not significantly affect the growth of *L. sakei* CTC494 compared to monoculture conditions, but affected the growth capability of *L. monocytogenes* CTC1034, mainly by reducing its maximum population density (N_{max}), especially with increasing the initial concentration of the bioprotective strain and lowering the storage temperature (Table 3). In accordance with the observed results, the fit of the squared root model to the μ_{max} values for *L. sakei* CTC494 and *L. monocytogenes* CTC1034 obtained in coculture conditions was not statistically different from that obtained with the fitting of the model to the observed μ_{max} in monoculture conditions (Table 4). Thus, the growth rate of *L. monocytogenes* was not significantly affected by the presence of *L. sakei* CTC494. On the other hand, while estimates of the maximum population density (N_{max}) for *L. sakei* CTC494 were not affected by either the temperature or the presence of the pathogen with a mean value of 7.72 ± 0.44 Log cfu/g, for *L. monocytogenes* a significant effect of the temperature was observed. In particular, a second degree polynomial model for each ratio was needed to properly describe the effect of temperature on the N_{max} of *L. monocytogenes* (Table 4).

Regarding the Jameson-effect model with interaction *γ* factor, for temperatures > 5 °C the *γ* parameter was not significantly affected by the storage temperature, which is in agreement with the findings of Mejlholm & Dalgaard (2015) on the simultaneous growth of lactobacilli and *L. monocytogenes* in brined shrimp or mayonnaise-based shrimp salad at 5 – 12 °C. For modelling purposes, *γ* parameter was equal to 1 for temperatures >5°C. On the other hand, at 2°C the estimated *γ* values for ratios 10:10, $10:10^3$ and $10:10^5$ were <1, very close to 1 and higher than 1, respectively (Table 3). These results suggested that at low temperatures (< 5 °C) *γ* could be dependent on temperature.

3.5 Simulation and evaluation of the developed interaction model under non-isothermal conditions.

To evaluate the performance of the developed interaction model, independent data about the simultaneous growth of *L. sakei* CTC494 and *L. monocytogenes* CTC1034 in cooked ham at 3 different pathogen-bioprotective ratios (10:10, $10:10^3$ and $10:10^5$) obtained during the storage of cooked ham at 2 non-isothermal profiles (profile 1: 2.4 - 9.1 °C and profile 2: 0 - 20 °C) were used (Figure 3).

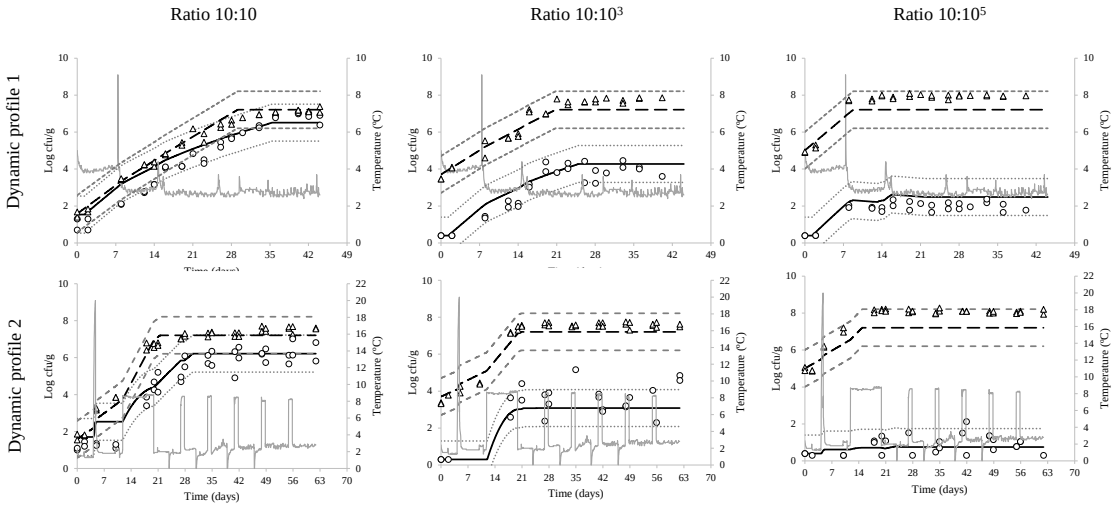

Figure 3. Observed datapoints of *L. sakei* CTC494 (triangles) and *L. monocytogenes* CTC1034 (circles) in cooked ham with 3 different initial concentrations of *L. sakei*, leading to *L. monocytogenes*:*L. sakei* ratios of 10:10, $10:10^3$ and $10:10^5$ cfu/g and stored at 2 different dynamic temperature profiles. Dashed and continuous black lines correspond to the predictions of the modified Jameson-effect with interaction *γ* model developed in the present study. Dashed and dotted grey lines correspond to the acceptable simulation zone (ASZ) for *L. sakei* and *L. monocytogenes*, respectively, used to compare the observations and predictions. Grey continuous lines stand for the storage temperature recorded.

The temperatures of both dynamic profiles were below 5 °C during most of the storage time. Within this low temperature range (i.e. 0 to 5 °C), for the ratio 10:10, *L. monocytogenes* growth was observed after *L. sakei* CTC494 reached its N_{max} in the dynamic profiles 1 and 2, confirming the temperature dependence of *γ* at low temperature.

The Jameson-effect model with interaction *γ* factor was used to carry out simulations. Kinetic parameters (secondary models and point-estimate values) and interaction *γ* factors estimated previously were tested. Due to the temperature dependence of *γ* at low temperatures, mentioned above, a linear effect of temperature on the *γ* values from 5 °C to 2 °C was assumed for model simulation. In the case of N_{max} , the use of the average from all temperatures under monoculture conditions (8.02 log CFU/g) showed better results, and therefore, this value was used instead of the secondary model including the influence of temperature (Eq 8). Regarding the growth rate, the secondary model obtained for coculture data provided the best results. The simulation with the model showed a good performance (Figure 3) in both non-isothermal profiles, with ≥ 81% of the predicted values being within the ASZ (± 1 Log) (Table 5).

Table 5. Percentage of *L. sakei* CTC494 and *L. monocytogenes* Log counts predicted within the ASZ for the non-isothermal profiles 1 and 2.

Non-isothermal profile	Ratio *Lm*:*Ls*[a]	*L. sakei* CTC494	*L. monocytogenes* CTC1034
1	10:10	100	81
	$10:10^3$	100	93
	$10:10^5$	100	100
2	10:10	100	81
	$10:10^3$	100	83
	$10:10^5$	91	96

[a]*Lm*: *L. monocytogenes*; *Ls*:*L. sakei* CTC494

Overall, results validated the predictive capacity of the developed mathematical model describing the antilisteria bioprotective effect of *L. sakei* CTC494 during the cooked ham shelf-life, even when exposed to abusive temperatures of storage. The developed model allowed to quantitatively characterize the antilisteria effect of *L. sakei* CTC494 on the N_{max} of *L. monocytogenes* throughout the modified Jameson-effect model with interaction □ factor to properly describe the temperature-dependent effect on the *γ*

parameter at chill temperatures (< 5°C). Moreover, the application of the predictive model constitutes an useful approach that could be used by food manufacturers to control and optimize the use of the *L. sakei* CTC494 strain as a bioprotective culture, e.g. to determine the concentration of *L. sakei* CTC494 to be added in the product according to foreseeable storage temperature to accomplish with the food safety legislation and/or to extend the safe shelf-life of the product.

4 Conclusions

The new quantitative evidences reported in the present work for the antilisteria strain *L. sakei* CTC494 makes this bioprotective culture a feasible food preservation strategy that can be used by food manufacturers to control *L. monocytogenes* growth in vacuum-packed cooked ham stored under isothermal and non-isothermal conditions, including abusive temperatures. In this sense, the new mathematical model developed in this study could be used by food manufacturers as a useful approach to optimize the conditions of use of the bioprotective culture with the aim to extend the safe shelf-life of the product.

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7 Supplementary data

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Article 10

Key factors determining the behaviour of pathogens in dry-cured ham after high-pressure processing

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Key Factors Determining the Behavior of Pathogens in Dry-Cured Ham after High Pressure Processing

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Abstract: High pressure processing (HPP) inactivates pathogens and increases the safety of ready-to-eat meat products. The high-pressure lethality and the behavior of the surviving cells after HPP depends on process parameters (pressure and time), microorganism and matrix characteristics. The aim of the present study was to quantify the impact of pressure level, water activity (a_w), and fat content on the behavior of *Salmonella* spp. and *Listeria monocytogenes* during refrigerated storage of dry-cured ham after high-pressure processing. *Salmonella enterica* serotype London CTC1003 and *L. monocytogenes* CTC1034 were inoculated at ca. 7 log cfu/g in dry-cured ham of different a_w (0.87–0.98), vacuum packaged, pressurized from 300 to 852 MPa for 5 min, and stored at 7 °C for up to 2 months. *Salmonella* and *L. monocytogenes* populations were monitored by plate count during the storage of the hams. The gamma concept was used to quantify the individual effects of a_w and storage temperature on the pathogen growth/no-growth behavior in pressurized dry-cured ham. The Weibull (inactivation) or Logistic (growth) primary models were fitted to the log change of pathogen levels during storage of dry-cured ham after pressurization. According to the gamma approach, the refrigeration temperature and a_w were the main factors limiting the growth of *Salmonella* and *L. monocytogenes*, respectively, in dry-cured ham. Under conditions not allowing growth, the effect of increasing pressures on the microbial inactivation depended on the a_w of dry-cured ham and the pathogen; dry-cured ham with high fat content with an $a_w \geq 0.95$ enhanced the inactivation of *Salmonella* whereas it reduced that of *L. monocytogenes*. Under conditions allowing growth of *L. monocytogenes*, the increase in a_w from 0.96 to 0.98 reduced the lag time with no apparent impact on the growth rate.

Keywords: shelf-life; food safety; *Listeria monocytogenes*; *Salmonella*; non-thermal inactivation; ready-to-eat meat products; high hydrostatic pressure

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1. Introduction

High pressure processing (HPP) is a non-thermal technology with an increasing implementation in the food industry used to enhance microbiological safety and/or render products with extended shelf-life. HPP is widely applied in meat products, representing 20–30% of overall pressurized products in the market [1]. In particular, HPP is usually used to inactivate pathogens such as *Salmonella* and *Listeria monocytogenes* in ready-to-eat (RTE) meat products once they are packed as blocks or diced or sliced convenience products. The efficacy of HPP in reducing microbial loads makes this technology particularly interesting for food-business operators to assure the accomplishment of regulations where no detection of pathogens such as *Salmonella* in RTE meat products is required [2]. Moreover, the application of HPP can be very useful to control *L. monocytogenes* in products intended to

be marketed under the umbrella of the zero-tolerance policy [3]. Within RTE meat products, dry-cured ham (DCH) is formulated with curing salts as preservatives and subsequently dried to water activity (a_w) below 0.92, which leads to DCH being considered a shelf-stable product, i.e., pathogenic microorganisms such as *Salmonella* and *L. monocytogenes* cannot grow [4–7]. However, a survey conducted on retail products of sliced and pre-packed DCH showed that 50% of the samples had an a_w above 0.92 [8]. Therefore, shelf-stability may not always be assured in terms of complying with food-safety microbiological criteria since pathogens contaminating the product may survive or even grow at a high a_w during storage. In these cases, the application of in-package lethality treatments may be needed to reduce the microbial load before storage. One of the drawbacks faced by RTE meat manufacturers when applying HPP is the enhanced pressure resistance of microorganisms in products with a low a_w, especially for products with an $a_w \leq 0.92$ such as DCH [9–11]. Therefore, the benefits in terms of pathogen-growth restriction provided by the intrinsic characteristics of DCH can turn into a limitation when applying HPP as an in-package lethality treatment aiming to eliminate pathogenic bacteria. Besides the protection of a low a_w on HPP lethality, the effects of other DCH constituents on pressure resistance and particularly the subsequent behavior of surviving cells has scarcely been evaluated. Bover-Cid et al. [9,12] studied the effect of a_w, fat, and pressure on the HPP lethality of *Salmonella* and *L. monocytogenes* in DCH. The results of these studies showed that the HPP lethality of both pathogens increased with increasing pressure and a_w of DCH. An increase in fat content did not significantly affect the lethality of *Salmonella* by HPP [12], whereas it led to a protective effect above 700 MPa for *L. monocytogenes* [9]. However, these studies did not report the influence of a_w and fat content of the DCH nor the pressure level on the subsequent behavior of *Salmonella* and *L. monocytogenes* during the storage of pressurized DCH.

Predictive microbiology, also known as quantitative microbial ecology, can be used to characterize the effect of different factors on the behavior of microorganisms in food [13]. In particular, the gamma-concept approach accounts for the individual effects of intrinsic (a_w, pH, and lactic acid) and extrinsic (storage temperature) factors and their interaction on the pathogen-growth behavior [14,15]. The aim of the present study was to continue and expand previous work on the effect of HPP on the lethality (as immediate inactivation) of *L. monocytogenes* and *Salmonella* in DCH [9,12], gaining more knowledge on the impact of the a_w and fat content of DCH and pressure level on the subsequent behavior of *Salmonella* and *L. monocytogenes* during the refrigerated storage of DCH after an HPP treatment. To do so, a quantitative study based on the determination of kinetic parameters was used to characterize the behavior of *Salmonella* and *L. monocytogenes* in DCH after HPP.

2. Material and Methods

2.1. Bacterial Strains

The bacterial strains used in the present study were *Salmonella* serovar London CTC1003 and *L. monocytogenes* strain CTC1034. Both strains were originally isolated from dry-cured meat products and have been used in previous studies dealing with the application of HPP in meat products [9,12]. The inoculums were prepared as in Bover-Cid et al. [9]. Briefly, stock cultures (stored in 20% glycerol at −80 °C) were transferred to 10 mL Brain Heart Infusion (BHI, from DB, NJ, USA) broth and incubated at 37 °C for 7 h. A second subculture was performed by transferring the first culture into a second tube of BHI and incubated at 37 °C for 18 h. An appropriate volume of this overnight culture was properly diluted to finally obtain a high inoculum level of ca. 10^7 cfu/g in DCH to ensure quantifiable levels were obtained after HPP.

2.2. Experimental Designs and Preparation of the Samples

The experimental layout of the experimental designs is shown in Figure 1. Firstly, a central composite design (CCD) with three variables (a_w, fat content, and pressure) and five levels, with an a_w ranging from 0.86–0.94, fat from 10–50%, and pressure level from 347–852 MPa, as described in Bover-Cid et al. [9,12], was followed. Secondly, to characterize

subsequent behavior of the pathogens under DCH a_w values around the growth/no-growth interface, a full factorial design (FFD) with 2 variables (a_w and pressure) was conducted in DCH with an a_w of 0.94–0.98 and with a fixed fat content of 30%. The factors and levels of the design were selected to cover a wide range of physicochemical characteristics of DCH [16].

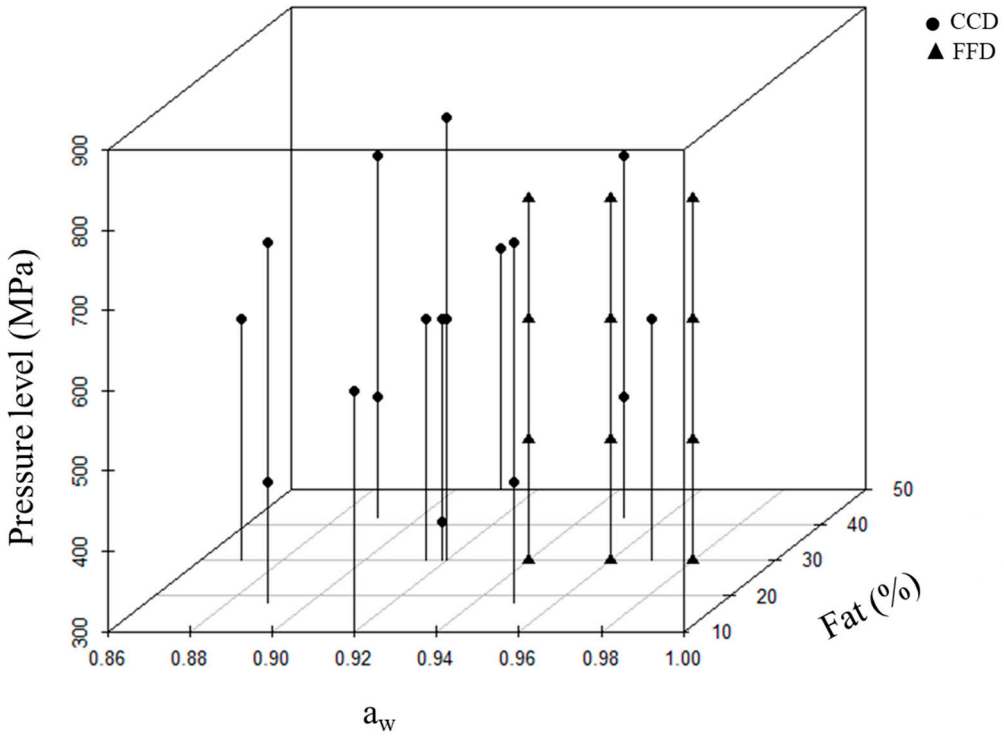

Figure 1. Scatterplot showing the a_w and fat content of the dry-cured ham (DCH) and pressure levels included in the experimental conditions of the central composite design (CCD) and the full factorial design (FFD).

DCHs were aseptically deboned in the laboratory. The lean part (with an a_w of 0.85 and 6.7% fat) was aseptically separated from the fat part, and each part was separately minced under aseptic conditions (minced lean showing pH 5.7 and 5000 ppm of water-phase lactic acid from endogenous origin). DCH matrices with the a_w and fat content adjusted in accordance with the target values of the experimental-design trials were prepared as described in Bover-Cid et al. [9,12]. Briefly, to adjust the a_w of the product, the appropriate volume of distilled water was added to the minced lean part, mixed, and equalized until homogenization to reach the target a_w. The inoculum was added to the distilled water immediately before mixing with the minced lean part. Afterwards, the proper quantity of minced fat corresponding to each trial was added to the inoculated lean samples. The actual a_w of the samples was verified with Aqualab^{TM} equipment (Series 3, Decagon Devices Inc., Pullman, WA, USA). The DCH was distributed in 15 g-samples and vacuum-packaged in PET/PE plastic bags (with oxygen permeability < 50 $cm^3/m^2/24$ h and water vapor permeability < 15 $mg/m^2/24$ h; Sacoliva S.L., Barcelona, Spain). For each trial, two DCH sample replicates were prepared.

2.3. HPP and Subsequent Storage of DCH

Samples were pressurized at the target pressure according to the corresponding trial of the experimental designs, which were in the range of 347 to 852 MPa. All treatments were applied for 5 min and with an initial fluid temperature of 15 °C. For pressures up to 600 MPa, Wave 6000 Hiperbaric (Burgos, Spain) equipment was used. For pressures above 600 MPa, Thiot ingeniere—Hiperbaric (Bretenoux, France—Burgos, Spain) equipment was

used. The pressure come-up rate was on average 220 MPa/min and pressure release was almost immediate (<2 s). After HPP, samples were stored at 7 °C for up to 60 days and were periodically taken for bacterial enumeration. Samples were stored at 7 °C as recommended and at foreseeable storage conditions for ready-to-eat meat products [17–19].

2.4. Microbiological Analysis

Samples were homogenized (1/10 dilution) with tryptic soy broth with 0.6% yeast extract (TSBYE; DB, NJ, USA) in a Masticator Classic (IUL S.A., Barcelona, Spain) for 1 min and subsequently 10-fold diluted in 0.1% Bacto Peptone (Difco Laboratories, Detroit, MI, USA) with 0.85% NaCl (Merck, Darmstadt, Germany). For the periodical enumeration of *Salmonella*, homogenates were plated on Brilliant Green Agar (BGA; Difco Laboratories, Detroit, MI, USA) and incubated at 37 °C for 24–48 h. For *L. monocytogenes*, homogenates were plated on the selective and differential medium Chromogenic Listeria Agar (CLA; Oxoid Basingstoke, UK) and incubated at 37 °C for 48 h. For expected counts below the limit of quantification, i.e., 4 cfu/g (resulting from platting 4 mL of homogenate in a 14 cm diameter plate), the presence of both pathogens in 15 g test samples was determined by enrichment of the homogenates at 37 °C for 48 h. The enriched homogenates were streaked on selective media (BGA for *Salmonella* and CLA for *L. monocytogenes*) and incubated at 37 °C for 24–48 h. Presumptive colonies were confirmed by PCR [20].

2.5. Statistical Analysis and Mathematical Modeling of the Pathogen Behavior during the Storage of the DCH after the HPP

The statistical analysis and mathematical modeling were conducted on data transformed into log change (log N/N_0), i.e., the decimal logarithm of the pathogen concentration at each sampling point minus the concentration of the pathogen at the beginning of the storage (immediately after the HPP). Values of the log change > 0.5 log units were considered growth behavior, log changes <-0.5 log were considered inactivation behavior, and log changes between -0.5 and 0.5 were considered not microbiologically relevant changes [18] and are termed "survival" in this article.

Principal component analysis (PCA) was conducted to provide a general overview of the dynamics along the storage of the pathogen in terms of changes in the concentration (log change) after HPP for the different combination of conditions. To perform the PCA, the *estim_ncpPCA* and *imputePCA* functions from the *missMDA* package of R software [21] were used to deal with the missing values, i.e., when the pathogen was not detected. The PCA with the confidence ellipses around the categoric variables (a_w, fat, and pressure level) at a confidence level of 0.95 was obtained with the *PCA* and *plotellipses* functions from the *FactoMinerR* package of R software [21]. Statistical differences in microbial log change (log N/N_0) along the storage time between trials were assessed through an ANOVA test followed by a Tukey's honestly significant difference test. For this, the *aov* function from the *stats* package and the *TukeyHSD* function from the *agricolae* package of the R software [21] were used.

2.5.1. Estimation of Growth/No-Growth Behavior

To assess whether the experimental environmental conditions (i.e., intrinsic parameters of DCH and storage temperature) of each trial would support the growth of *L. monocytogenes* and *Salmonella* during the storage of the DCH after HPP, the gamma-concept approach was applied [14,15,22]. The overall effect of the combination of the most relevant environmental factors influencing the growth (i.e., intrinsic: a_w, pH, lactic-acid concentration; and extrinsic: storage temperature) was estimated by calculating the overall gamma product (Γ. Equation (1)), including the interaction factor.

$$\Gamma = \prod_{i=1}^{k} \gamma_X(X_i) \cdot \xi \quad (1)$$

where (X_i) is defined by the physico-chemical properties of the ham during the storage (e.g., pH, a_w, lactic acid, and temperature). The individual effect of each environmental factor (X) on the pathogen growth is described by the individual gamma factor γ_X, whereas ξ is the interaction between factors. The γ_X and ξ values can vary from 0 to 1, with 0 indicating that growth is depleted by the environmental factor at a level of X_i or the interaction ξ and 1 indicating that the growth potential is optimal for this particular environmental factor [15]. The detailed procedure and cardinal values used for the calculation of γ_X and ξ values are described in Figure S1 and Table S1.

The growth behavior was defined based on the overall product of gamma factors with their interaction, being considered no growth when the output of Equation (1) was zero ($\Gamma = 0$) and growth when the model output was higher than zero ($\Gamma > 0$) [15]. For no-growth conditions ($\Gamma = 0$) the inactivation behavior was further explored according to Section 2.5.2, whereas when growth conditions were observed the growth kinetic parameters were characterized according to Section 2.5.3.

2.5.2. Non-Thermal Inactivation Kinetic Parameters during the Storage of DCH after HPP

The Weibull model (Equation (2)) was used to estimate the kinetic parameters describing the non-thermal inactivation of the pathogens during the refrigerated storage of DCH after HPP.

$$log\left(\frac{N}{N_0}\right) = -\left(\frac{t}{\delta}\right)^p \quad (2)$$

where N is the number of cells at time t and N_0 is the number of cells at the beginning of the storage time; $log\ (N/N_0)$ is the inactivation in log reduction (log units) at a given time (t) of the storage, being equal to 0 at storage time 0; δ is the time (days) necessary to obtain the first log reduction; and p is the shape parameter. Model fitting was carried out using the *nls2* package of the R software [21].

2.5.3. Estimation of Growth Kinetic Parameters during the Storage of DCH after HPP

The primary Logistic growth model with delay (Equation (3), [23]) was used to estimate the growth kinetic parameters of the pathogens during the refrigerated storage of DCH after HPP. The model was fitted to the log change data (i.e., log N_t/N_0) using the *nls2* package of the R software [21].

$$\begin{gathered} \text{For } t < \lambda,\ \log\left(\frac{N_t}{N_0}\right) = 0 \\ \text{For } t \geq \lambda,\ \log\left(\frac{N_t}{N_0}\right) = \log\left(\frac{MGP}{1+\left(\frac{MGP}{N_0}-1\right)\cdot(\exp(-\mu_{max}\cdot(t-\lambda)))}\right) \end{gathered} \quad (3)$$

where N_0 is the concentration of the pathogen (cfu/g) at time zero, N_t is the concentration of the pathogen (cfu/g) at a given time (t), MGP is the maximum growth potential (maximum bacterial increase in log units), λ is the lag time (days); μ_{max} is the maximum specific growth rate (h^{-1}), and t is the storage time (days).

3. Results and Discussion

3.1. Salmonella Behavior in DCH during Storage after HPP

The results of the challenge test showed that *Salmonella* was not able to grow in any of the conditions assessed (Figure 2). The results agreed with those obtained through the application of the gamma concept, accounting for the individual effects of intrinsic (a_w, pH, and lactic acid) and extrinsic (storage temperature) factors and their interaction on *Salmonella* behavior, where no growth ($\Gamma = 0$) was predicted to occur in DCH with a $a_w < 0.98$ (Table 1). Growth of *Salmonella* was predicted at $a_w = 0.98$ but the Γ value was very close to zero ($\Gamma = 0.0002$; trials 29–32; Table 1). The main contributing growth-inhibition factor was the storage temperature ($\gamma_T = 0.005$, Table S2) since the storage temperature applied (7 °C) was close to the minimum growth temperature of *Salmonella* (Table S1). Results of challenge test showed that under the non-growing conditions, the viability of *Salmonella*

was compromised in all the trials, resulting in a progressive log reduction (inactivation) of the pathogen load along the refrigerated storage (Figure 2, Table 1). The results of the PCA analysis showed that 99.73% of the variability could be represented in a two-dimensional space (Figure 3). The same length of the arrows represented in the correlation circle shows that all the sampling times contributed highly to a similar extent to explaining the variability in the *Salmonella* log change data during storage (Figure 3a). In dimension 1, the horizontal axis explained 92.58% of the variability of the *Salmonella* log change data and all the arrows pointed in the same direction, indicating a high correlation among sampling times. The confidence ellipses grouped trials with pressure < 600 MPa on the right and trials with pressure > 600 MPa on the left of the PCA graph (Figure 3d), suggesting an important impact of pressure level on *Salmonella* inactivation along the storage of DCH, which could be related to the high lethality of > 600 MPa treatments.

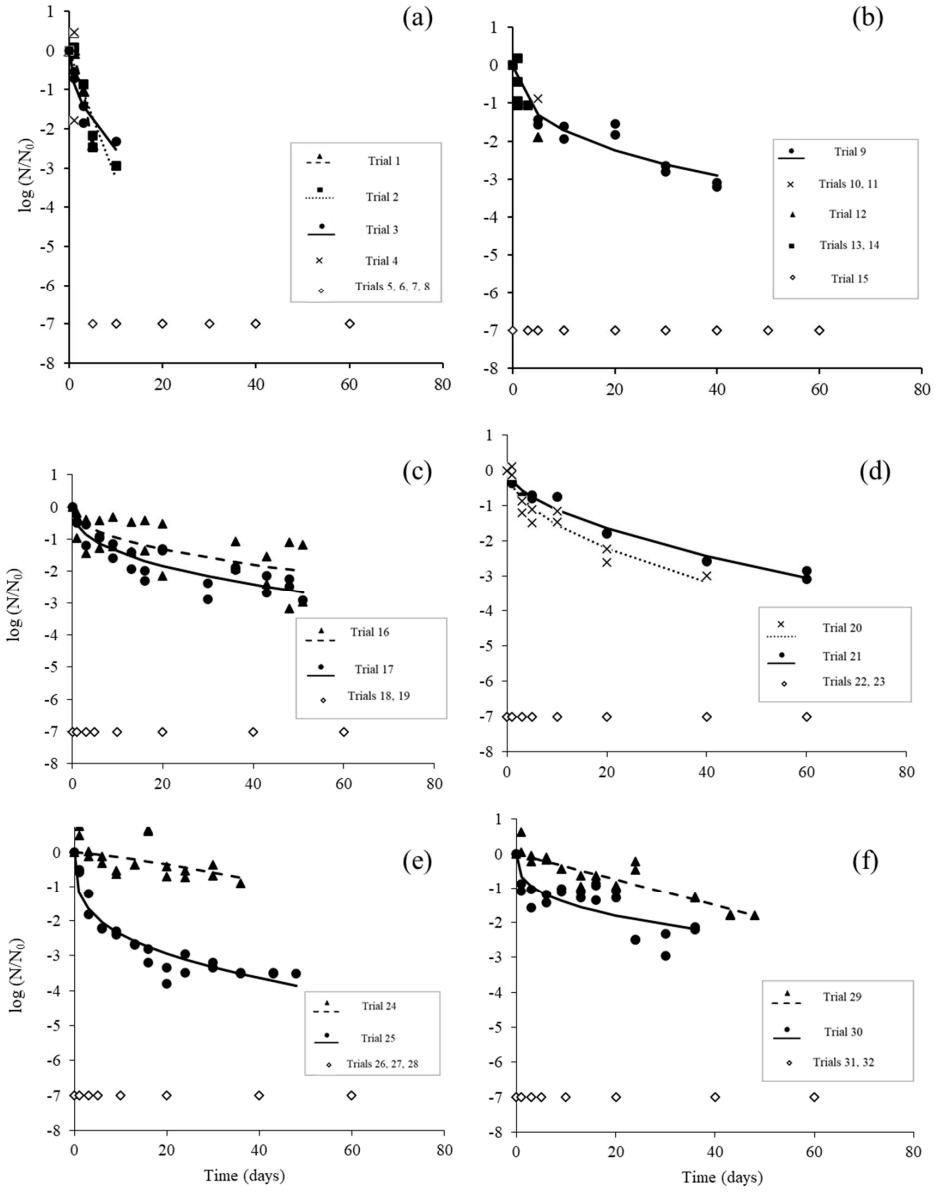

Figure 2. *Salmonella* survival and inactivation during storage at 7 °C of pressurized dry-cured ham (DCH) with an a_w of (**a**) 0.870–0.915, (**b**) 0.919–0.920, (**c**) 0.940, (**d**) 0.950, (**e**) 0.960, and (**f**) 0.980. Symbols correspond to log change values and lines to the fit of the Weibull model (Equation (2)) to the data. Empty symbols correspond to replicates where the pathogen was not detected.

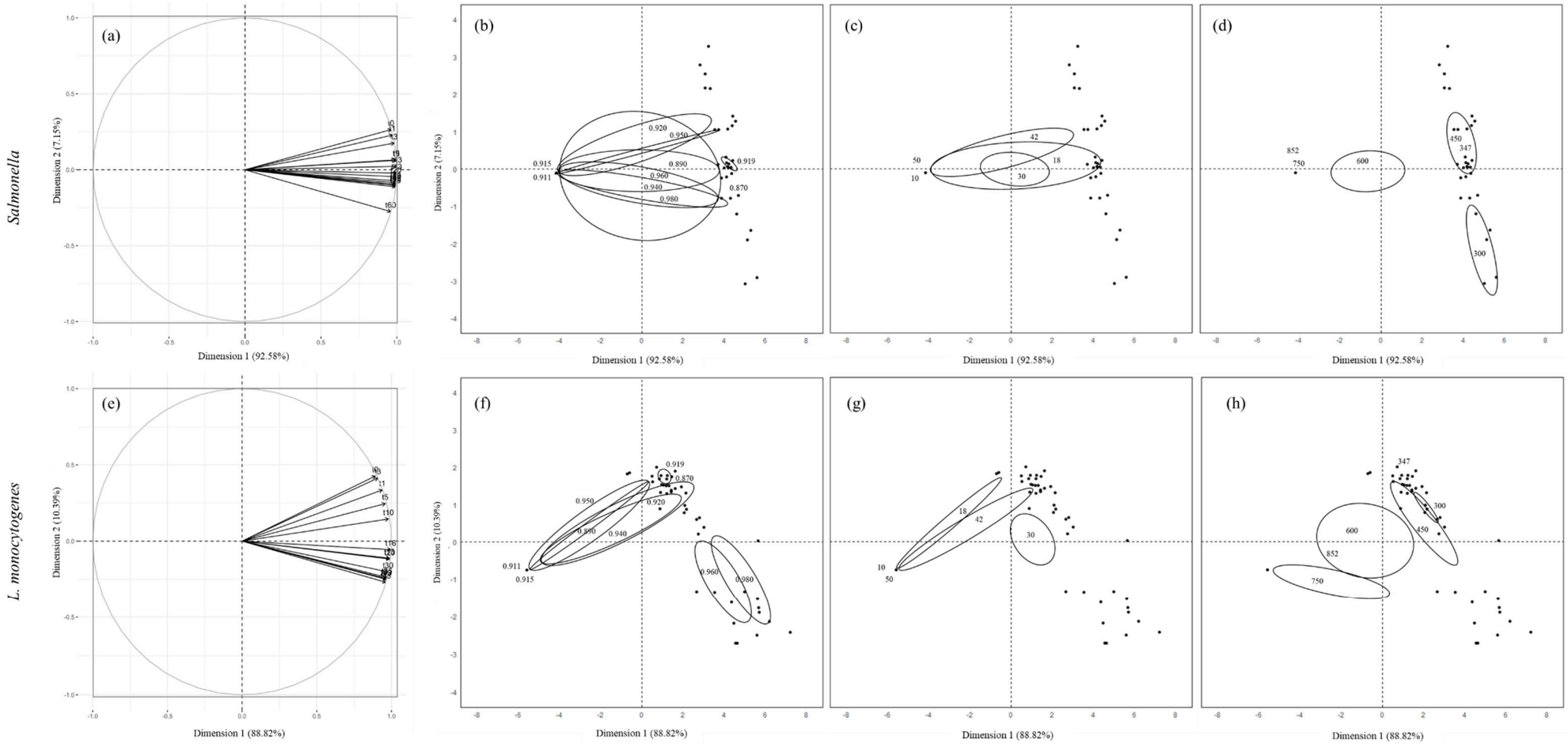

Figure 3. Results of the principal component analysis (PCA) performed on the log change values of *Salmonella* (**a**–**d**) and *L. monocytogenes* (**e**–**h**) observed during the storage of pressurized dry-cured ham (DCH) at 7 °C. (**a**,**e**) show the correlation circles with the relationship between all the sampling times (days) on the log change data of *Salmonella* and *L. monocytogenes*. In (**b**–**d**,**f**–**h**), the points represent the scores for the first two dimensions of PCA corresponding to each replicate of the 32 trials performed and the ellipses correspond to confidence ellipses at a confidence level of 0.95 around the categoric variables a_w (**b**,**f**), % of fat content (**c**,**g**), and pressure level in MPa (**d**,**h**).

Table 1. *Salmonella* concentration immediately after high pressure processing (HPP) in the different trials and estimated kinetic-parameter values resulting from fitting the primary inactivation model to the *Salmonella* inactivation counts observed during the storage of pressurized dry-cured ham (DCH) at 7 °C.

Trial	DCH Characteristics		HPP (MPa)	Concentration after HPP (log cfu/g) [a]	Observed Behavior during Storage [b]	Predicted G/NG (Γ) [d]	Inactivation Kinetic Parameters [e]		Goodness of Fit [f]		
	a_w	Fat (%)					δ (Days)	p	n	par	RMSE
1	0.870	30	600	3.71 ± 0.04	I	0 (NG)	3.03 ± 0.10	1.80 ± 0.13	7	2	0.091
2	0.890	18	450	3.55 ± 0.17	I	0 (NG)	2.90 ± 0.76	0.95 ± 0.25	9	2	0.538
3	0.890	42	450	2.92 ± 0.11	I	0 (NG)	1.48 ± 0.35	0.49 ± 0.08	7	2	0.215
4	0.890	18	750	2.69 ± 0.79	I [c]	0 (NG)	-	-	-	-	-
5	0.890	42	750	0.90 ± 0.43	I [c]	0 (NG)	-	-	-	-	-
6	0.911	50	600	1.66 ± 0.26	I [c]	0 (NG)	-	-	-	-	-
7	0.915	30	600	1.39 ± 0.55	I [c]	0 (NG)	-	-	-	-	-
8	0.915	30	600	1.80 ± 0.14	I [c]	0 (NG)	-	-	-	-	-
9	0.919	30	347	5.76 ± 0.17	I	0 (NG)	2.43 ± 1.07	0.38 ± 0.06	16	2	0.365
10	0.919	30	600	2.78 ± 0.07	I [c]	0 (NG)	-	-	-	-	-
11	0.919	30	600	3.03 ± 0.20	I [c]	0 (NG)	-	-	-	-	-
12	0.920	10	600	2.57 ± 0.22	I [c]	0 (NG)	-	-	-	-	-
13	0.920	30	600	2.72 ± 0.22	I [c]	0 (NG)	-	-	-	-	-
14	0.920	30	600	2.38 ± 0.66	I [c]	0 (NG)	-	-	-	-	-
15	0.920	30	852	<DL	K	0 (NG)	-	-	-	-	-
16	0.940	30	300	7.10 ± 0.63	I	0 (NG)	10.76 ± 4.31	0.44 ± 0.14	24	2	0.643
17	0.940	30	450	4.74 ± 0.03	I	0 (NG)	4.56 ± 1.23	0.41 ± 0.06	26	2	0.356
18	0.940	30	600	<DL	K	0 (NG)	-	-	-	-	-
19	0.940	30	750	<DL	K	0 (NG)	-	-	-	-	-
20	0.950	18	450	4.64 ± 0.03	I	0 (NG)	8.18 ± 0.96	0.56 ± 0.04	16	2	0.186
21	0.950	42	450	3.70 ± 0.15	I	0 (NG)	4.42 ± 0.98	0.52 ± 0.07	13	2	0.316
22	0.950	18	750	<DL	K	0 (NG)	-	-	-	-	-
23	0.950	42	750	<DL	K	0 (NG)	-	-	-	-	-
24	0.960	30	300	6.48 ± 0.05	I	0 (NG)	46.06 ± 5.97	1.24 ± 0.43	25	2	0.409
25	0.960	30	450	4.40 ± 0.01	I	0 (NG)	0.59 ± 0.23	0.31 ± 0.03	26	2	0.336
26	0.960	30	600	<DL	K	0 (NG)	-	-	-	-	-
27	0.960	30	600	<DL	K	0 (NG)	-	-	-	-	-
28	0.960	30	750	<DL	K	0 (NG)	-	-	-	-	-
29	0.980	30	300	6.30 ± 0.01	I	2.16×10^{-4} (G)	26.75 ± 1.98	1.00 ± 0.15	24	2	0.267
30	0.980	30	450	3.63 ± 0.09	I	2.16×10^{-4} (G)	3.48 ± 1.66	0.33 ± 0.09	22	2	0.457
31	0.980	30	600	<DL	K	2.16×10^{-4} (G)	-	-	-	-	-
32	0.980	30	750	<DL	K	2.16×10^{-4} (G)	-	-	-	-	-

[a] Mean ± standard deviation of *Salmonella* concentration immediately after HPP. Conditions where the pathogen concentration after HPP was below the detection limit are indicated with <DL. [b] I: inactivation behavior (observed log change <−0.5 log units); K: the application of HPP resulted in the total inactivation of the pathogen in the sample unit and/or the pathogen was not able to recover viability during storage. [c] Few quantification points before *Salmonella* was inactivated to concentrations below the detection limit or no detection of the pathogen during storage. Kinetic parameters could not be estimated. [d] Growth/no-growth (G/NG) boundary as predicted by the gamma concept (*Γ*). For each DCH with a different a_w value, *Γ* was calculated considering a storage temperature of 7 °C, pH of DCH of 5.7, and lactic-acid content of DCH of 5000 ppm in the water phase. [e] The Weibull model was fitted to log change data (Equation (2)) to estimate the inactivation kinetic parameters. *δ*: time (days) for the first log reduction during storage; *p*: shape of the inactivation curve. The estimate ± standard error is provided. [f] n: number of data points ($\log N/N_0$) included for fitting; par: parameters estimated in the model; RMSE: root mean square error.

Specifically, the pressurization of DCH at ≥ 600 MPa resulted in a lethality of *Salmonella* to levels below the detection limit in DCH with an a_w > 0.92 from immediately after HPP to the end of the storage (trials 18–19, 22–23, 26–28, 31–32; Table 1). In DCH with an $a_w \leq 0.92$ (trials 4–8, 10–14; Table 1), due to the protective effect of a low a_w in front of HPP or piezo-protection [12], *Salmonella* could be enumerated. However, after HPP the remaining levels were low and decreased below the detection limit just after 1 day of storage, thus not allowing for the estimation of inactivation kinetic parameters (trials 4–8, 10–14; Table 1). In this framework, Stollewerk et al. [24] observed that the levels of *Salmonella* after the application of HPP at 600 MPa for 5 min in smoked DCH (pH of 5.87 and a_w of 0.93) were low and progressively decreased below the detection limit after the storage of DCH at 4 °C for 38 days and afterwards at 8 °C for 18 days. The longer survival of *Salmonella* in DCH observed by Stollewerk et al. [24] compared to the results obtained in the present study could be partially attributed to the lower storage temperature. In this respect, Serra-Castelló et al. [6] quantified that the storage of DCH at 4 °C favors the survival of *Salmonella* in non-pressurized DCH compared to the storage of the product at 7 °C. On the other hand, the application of pressure levels < 600 MPa led to *Salmonella* concentrations above the detection limit, allowing inactivation kinetics to be characterized along the storage of the DCH with different intrinsic characteristics (a_w and fat) (trials 1–3, 9, 16–17, 20–21, 24–25, 29–30; Table 1; Figure 2).

The increase in the pressure level applied from 300 to 450 MPa resulted in enhanced *Salmonella* inactivation (shorter δ parameter) during the refrigerated storage of DCH (trials 16–17, 24–25 and 29–30; Table 1; Figure 2), though its impact on the *Salmonella* inactivation kinetics curve was only statistically significant (p-value < 0.05) in DCH with an $a_w \geq 0.96$ (trials 24–25, 29–30; Figure 2). A higher content of fat (42%) in DCH seemed to enhance the inactivation of the pathogen during the refrigerated storage after HPP in DCH with an a_w of 0.89 and especially with an a_w of 0.95 (trials 2–3, 20–21; Table 1), though it was not statistically significant.

3.2. *L. monocytogenes* Behavior in DCH during Storage after HPP: Growth/No Growth

The results of challenge test showed that *L. monocytogenes* was able to grow in DCH with an a_w of 0.96 regardless of the HPP level applied, whereas growth was not observed in DCH with a lower a_w (Figure 4). Contrary to *Salmonella*, the output of the gamma approach showed that temperature was not the main limiting factor for the pathogen's growth (γ_T = 0.055, Table S2), as *L. monocytogenes* is a psychrotrophic microorganism able to grow at temperatures slightly below 0 °C [25]. The quantification of the individual effects of the intrinsic (a_w, pH, and lactic acid concentration) and extrinsic (storage temperature) factors and their interactions on the *L. monocytogenes* behavior through the gamma concept showed that, within the experimental domain of the present study, the growth/no-growth boundary predictions for this pathogen depended on the a_w, with the DCH with an a_w value of 0.95 being the predicted boundary value for *L. monocytogenes* growth (Table 2). A value of 0.92 is considered the minimum growth limit for *L. monocytogenes* when no other stressing factors are present [25]. However, when a low a_w is combined with other factors such as low temperature, its bacteriostatic effect is enhanced and the minimum growth limit decreases. In this respect, the microbiological criteria for foodstuffs [2] established that foods with an a_w < 0.92 are automatically considered unable to support the growth of the pathogen. According to this regulation, other categories of products can also belong to this category, subject to scientific justification. The growth/no-growth model predicted growth in DCH with an a_w = 0.95 with a very small Γ value ($\Gamma = 3.14 \times 10^{-3}$) and no growth of *L. monocytogenes* was observed when it was pressurized at 450 or 750 MPa (Trials 52–55, Table 2), indicating that the growth/no-growth boundary of the pathogen in pressurized DCH was also limited by other factors not considered in the calculation of the Γ factor, which could also include the potential injury caused by HPP on *L. monocytogenes* cells. Therefore, the present study provides scientific evidence to justify that the pressurized

DCH with an a_w > 0.92, up 0.95, can belong to the category not supporting the growth of *L. monocytogenes*.

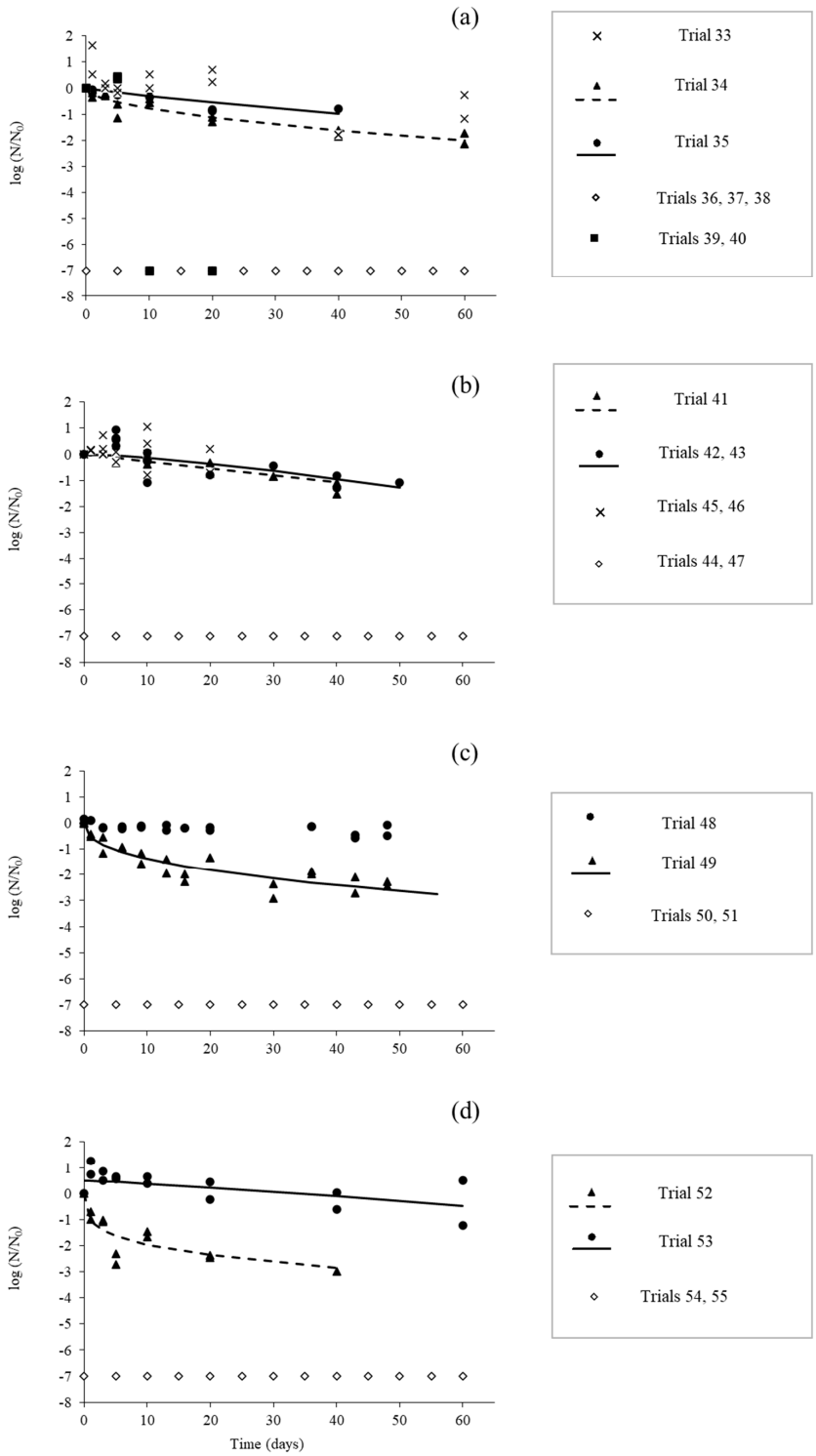

Figure 4. *L. monocytogenes* survival and inactivation during the storage at 7 °C of pressurized dry-cured ham (DCH) with an a_w (**a**) 0.870–0.915, (**b**) 0.919–0.920, (**c**) 0.940, and (**d**) 0.950. Symbols correspond to observed counts and lines to the fit of the Weibull model (Equation (2)) to data. Empty symbols correspond to replicates where the pathogen was not detected.

Table 2. *L. monocytogenes* concentration immediately after high pressure processing (HPP) in the different trials and estimated parameter values resulting from fitting the primary models to the *L. monocytogenes* inactivation/growth counts observed during the storage of pressurized dry-cured ham (DCH) at 7 °C.

Trial	DCH Characteristics		HPP (MPa)	Concentration after HPP (log cfu/g) [a]	Observed Behavior during Storage [b]	Predicted G/NG (Γ) [d]	Inactivation Parameters [e]		Growth Parameters [f]			Goodness of Fit [f,g]		
	a_w	Fat (%)					δ (Days)	p	λ (d)	μ_{max} (h^{-1})	MGP (log)	n	par	RMSE
33	0.870	30	600	1.78 ± 0.17	S	0 (NG)	-	-	-	-	-	-	-	-
34	0.890	18	450	4.34 ± 0.05	I	0 (NG)	15.74 ± 2.51	0.52 ± 0.08	-	-	-	15	2	0.251
35	0.890	42	450	4.69 ± 0.06	I	0 (NG)	40.28 ± 3.55	0.85 ± 0.15	-	-	-	15	2	0.191
36	0.890	18	750	<DL	K	0 (NG)	-	-	-	-	-	-	-	-
37	0.890	42	750	<DL	K	0 (NG)	-	-	-	-	-	-	-	-
38	0.911	50	600	<DL	K	0 (NG)	-	-	-	-	-	-	-	-
39	0.915	30	600	1.57 ± 0.38	I [c]	0 (NG)	-	-	-	-	-	-	-	-
40	0.915	30	600	1.81 ± 0.05	I [c]	0 (NG)			-	-	-			
41	0.919	30	347	5.83 ± 0.26	I	0 (NG)	37.57 ± 2.92	0.96 ± 0.19	-	-	-	14	2	0.227
42	0.919	30	600	1.96 ± 0.17	I	0 (NG)	41.84 ± 7.79	1.35 ± 0.80 [ns]	-	-	-	15	2	0.501
43	0.919	30	600	2.22 ± 0.12	I	0 (NG)			-	-	-			
44	0.920	10	600	<DL	K	0 (NG)	-	-	-	-	-	-	-	-
45	0.920	30	600	1.68 ± 0.17	I [c]	0 (NG)	-	-	-	-	-	-	-	-
46	0.920	30	600	1.15 ± 0.21	I [c]	0 (NG)			-	-	-			
47	0.920	30	852	<DL	K	0 (NG)	-	-	-	-	-	-	-	-
48	0.940	30	300	6.81 ± 0.12	S	0 (NG)	-	-	-	-	-	-	-	-
49	0.940	30	450	5.80 ± 0.02	I	0 (NG)	4.86 ± 1.17	0.40 ± 0.05	-	-	-	24	2	0.348
50	0.940	30	600	<DL	K	0 (NG)	-	-	-	-	-	-	-	-
51	0.940	30	750	<DL	K	0 (NG)	-	-	-	-	-	-	-	-
52	0.950	18	450	5.61 ± 0.07	I	3.14×10^{-3} (G)	0.81 ± 0.53	0.27 ± 0.05	-	-	-	15	2	0.432
53	0.950	42	450	2.15 ± 0.21	I	3.14×10^{-3} (G)	61.44 ± 28.77	1.18 ± 1.38 [ns]	-	-	-	15	2	0.792
54	0.950	18	750	<DL	K	3.14×10^{-3} (G)	-	-	-	-	-	-	-	-
55	0.950	42	750	<DL	K	3.14×10^{-3} (G)	-	-	-	-	-	-	-	-
56	0.960	30	300	7.02 ± 0.05	G	1.34×10^{-2} (G)	-	-	5.76 ± 1.55	0.011 ± 0.003	1.34 ± 0.08	26	3	0.142
57	0.960	30	450	2.10 ± 1.70	G	1.34×10^{-2} (G)	-	-	6.00 ± 2.97	0.038 ± 0.013	4.80 ± 0.40	24	3	1.644
58	0.960	30	600	<DL	G	1.34×10^{-2} (G)	-	-	NA	0.022 ± 0.003	7.05 ± 0.31	28	2	0.866
59	0.960	30	600	<DL	G	1.34×10^{-2} (G)								
60	0.960	30	750	<DL	G	1.34×10^{-2} (G)	-	-	NA	$0.088 \pm 7.541 \times 10^{3}$ [ns]	6.20 ± 0.71	12	2	1.560
61	0.980	30	300	6.81 ± 0.10	G	2.78×10^{-2} (G)	-	-	NA	0.021 ± 0.003	1.36 ± 0.04	22	2	0.163

Table 2. *Cont.*

Trial	DCH Characteristics		HPP (MPa)	Concentration after HPP (log cfu/g) [a]	Observed Behavior during Storage [b]	Predicted G/NG (Γ) [d]	Inactivation Parameters [e]		Growth Parameters [f]			Goodness of Fit [f,g]		
	a_w	Fat (%)					δ (Days)	p	λ (d)	μ_{max} (h^{-1})	*MGP* (log)	n	par	RMSE
62	0.980	30	450	0.98 ± 0.11	G	2.78×10^{-2} (G)	-	-	NA	0.033 ± 0.003	6.89 ± 0.19	22	2	0.526
63	0.980	30	600	<DL	G	2.78×10^{-2} (G)	-	-	NA	0.021 ± 0.002	6.95 ± 0.34	17	2	1.327
64	0.980	30	750	<DL	G	2.78×10^{-2} (G)	-	-	NA	0.039 ± 0.014	6.96 ± 0.74	13	2	0.744

[ns] Parameter estimates not statistically significant. [a] Mean ± standard deviation of *L. monocytogenes* concentration immediately after HPP. Conditions where the pathogen concentration after HPP was below the detection limit are indicated with <DL. [b] S: *L. monocytogenes* survived without growth or inactivation (observed log change between −0.5 and 0.5 log units); I: inactivation (observed log change < −0.5 log units); K: the application of HPP resulted in the total inactivation of the pathogen in the sample unit and/or the pathogen was not able to recover viability during the storage; G: growth (observed log change > 0.5 log units). [c] Few quantification points before *L. monocytogenes* inactivated to concentration below the detection limit or no detection of the pathogen during storage. Kinetic parameters could not be estimated. [d] Growth/no-growth (G/NG) boundary as predicted by the gamma concept (*Γ*). For each DCH with different a_w value, *Γ* was calculated considering a storage temperature of 7 °C, pH of DCH of 5.7, and lactic-acid content of DCH of 5000 ppm in the water phase. [e] For conditions not supporting growth that caused a loss of *L. monocytogenes* viability, i.e., inactivation, the Weibull model was fitted to log change data (Equation (2)) to estimate the inactivation kinetic parameters. δ: time (days) for the first log reduction during storage; p: shape of the inactivation curve. The estimate ± standard error is provided. [f] For conditions supporting growth, the logistic growth model without and with delay (Equation (3)) was fitted to log change data to estimate the growth kinetic parameters. λ is the lag time (days), μ_{max} is the maximum specific log increase rate (h^{-1}); MGP is the maximum growth potential (maximum bacterial increase in log units). The estimate ± standard error is provided. [g] n: number of log change data points (log N/N_0) included for fitting; par: parameters estimated in the model; RMSE: root mean square error.

The results of the PCA showed that 99.21% of the variance in the log change data of *L. monocytogenes* could be represented in a two-dimensional space (Figure 3). The same length of the arrows represented shows that all the sampling times highly contributed to some extent to explaining the variability in the *L. monocytogenes* log change data during storage (Figure 3e). In dimension 1, all the arrows pointed to the right, indicating that the sampling times were correlated. The confidence ellipses grouped trials with pressure < 600 MPa on the right and trials with pressure > 600 MPa on the left of the PCA graph (Figure 3h), suggesting an important impact of pressure level on *L. monocytogenes* inactivation along the storage of DCH. Moreover, confidence ellipses also grouped trials with an $a_w \geq 0.96$ and trials with an $a_w < 0.96$ (Figure 3f), which was correlated with the observed growth of the pathogen (log change > 0) in DCH with an $a_w \geq 0.96$ and no growth (log change ≤ 0) with an $a_w < 0.96$ (Table 2).

3.2.1. *L. monocytogenes* No-Growth Conditions: Survival and Inactivation during Storage

No growth of *L. monocytogenes* was predicted to occur ($\Gamma = 0$) in products with an $a_w < 0.95$ (Table 2), which agreed with the observed results, where survival (no microbiologically relevant change, trials 33 and 48) or inactivation of *L. monocytogenes* was observed during the storage of DCH (trials 34–47 and 49–51; Table 2; Figure 4).

Trials involving HPP at 750–852 MPa had a strong lethal effect (*L. monocytogenes* was not detected), not allowing subsequent monitoring (trials 36–38, 44–47, 50–51, 54–55; Table 2; Figure 4). For pressure levels < 600 MPa, the concentration of *L. monocytogenes* after the HPP and its subsequent behavior was affected by the pressure level applied as well as the DCH's characteristics (a_w value and fat content). In DCH with an a_w of 0.87 pressurized at 600 MPa (Trial 33; Table 2; Figure 4a) the levels of *L. monocytogenes* after HPP were variable and close to the quantification level, with no detection of the pathogen in 12.5% of the samples along the storage. Overall, no relevant change (survival without inactivation) can be associated with these trial conditions. The survival of *L. monocytogenes* could be the consequence of the piezo-protection effect exerted by low a_w on the lethality of the pathogen by HPP. Indeed, this would result in fewer injured *L. monocytogenes* cells due to its role in protein stabilization, which prevents protein denaturation and cell death during HPP [26], and which in turn could contribute to keeping the pathogen viability after HPP.

L. monocytogenes was also able to survive without any significant change during the storage of DCH with an a_w of 0.94 pressurized at 300 MPa (Trial 48; Table 2; Figure 4c). Compared to other trials, in this case, the survival of the pathogen could be favored by either (i) the high a_w value (0.94), since the application of a similar pressure level (347 MPa) in DCH with a lower a_w (0.919) resulted in the inactivation of the pathogen during the storage (Trial 41; Table 2; Figure 4b), and/or (ii) the lower pressure compared with trial 49 (at 450 MPa, a_w of 0.94) in which inactivation was observed. These hypotheses would be supported by Stollewerk et al. [24], showing that the levels of *L. monocytogenes* after the pressurization of smoked DCH (pH of 5.87 and a_w of 0.93) at 600 MPa for 5 min were low and progressively inactivated to limits below the quantification limit (0 log cfu/g) after the storage of the DCH at 4 °C for 38 days and afterwards at 8 °C for 18 days.

In DCH with an a_w of 0.919, *L. monocytogenes* decreased during the storage of DCH after HPP irrespective of the pressure level (from 347 to 600 MPa; trials 41, 42 and 43; Table 2; Figure 4b). Therefore, even if the pressure increase enhances the immediate lethality of *L. monocytogenes* during HPP, it has no effect on the inactivation of the pathogen during the subsequent storage after HPP in DCH with an a_w of 0.919.

Regarding the impact of fat, an increase from 18 to 42% did not significantly affect the *L. monocytogenes* lethality during HPP nor its subsequent inactivation after HPP in DCH with an a_w of 0.89 (trials 34–35; Table 2; Figure 4a). Contrarily, for DCH with an a_w of 0.95, a higher content of fat enhanced the lethality of *L. monocytogenes* by HPP and significantly reduced the inactivation of the pathogen during the subsequent storage (trials 52–53; Table 2; Figure 4d).

3.2.2. *L. monocytogenes* Growth during the Storage of DCH

In agreement with the observed results, *L. monocytogenes* growth was predicted to occur ($\Gamma > 0$) in products with $a_w \geq 0.96$ (trials 56–64; Table 2). *L. monocytogenes* was able to reach the maximum bacterial-population density (*ca.* 8 log cfu/g) even for DCH pressurized at 750 MPa (Figure 5).

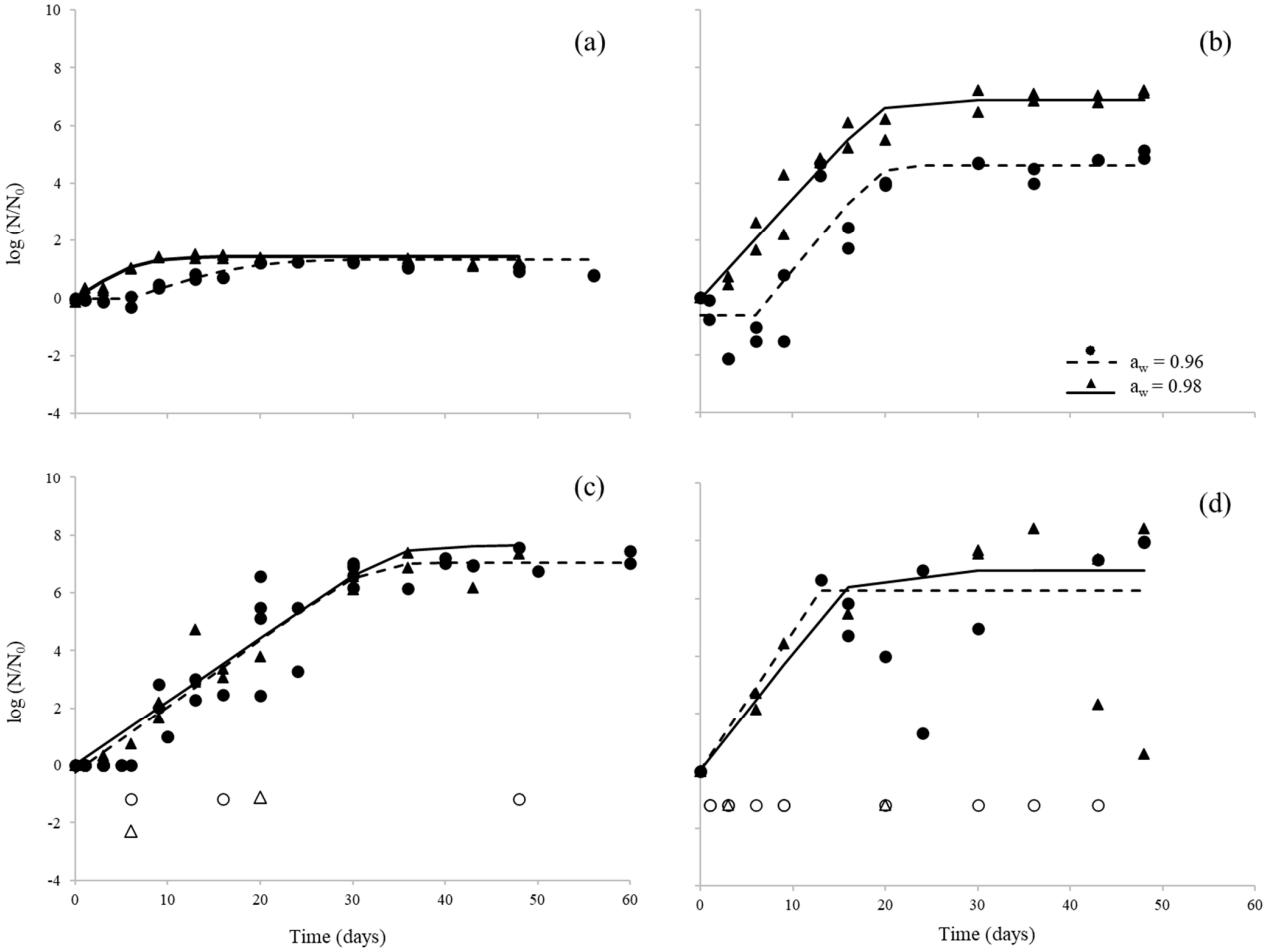

Figure 5. Growth of *L. monocytogenes* during storage at 7 °C of pressurized dry-cured ham (DCH) with an $a_w \geq 0.96$ pressurized at (**a**) 300 MPa (trials 56 and 61), (**b**) 450 MPa (trials 57 and 62), (**c**) 600 MPa (trials 58 and 63), and (**d**) 750 MPa (trials 59 and 64). Symbols correspond to log change values and lines to the fit of the Logistic growth model (Equation (3)) to data. Empty symbols correspond to replicates where the pathogen was not quantified but was detected after sample enrichment.

The growth kinetics were dependent on pressure level and DCH's a_w (which in turn determined the pathogen concentration at the beginning of the storage) (trials 56–64; Table 2). In this regard, the application of the lowest pressure level (300 MPa) caused little lethality. As a consequence, the high initial concentration (6.8–7.0 log cfu/g) of *L. monocytogenes* after HPP did not allow for proper estimation of the growth rate (trials 56, 61; Table 2; Figure 5a). The results show that DCH's a_w affected the lag time (λ) of *L. monocytogenes* in DCH pressurized at 300–450 MPa. The pathogen needed a λ of ca. 6 days before starting to grow in DCH with an a_w of 0.96 (trials 56–57; Table 2) but no λ was observed in DCH with an a_w of 0.98 (trials 61–62; Table 2). *L. monocytogenes* seemed to start growing immediately after the HPP at 600–750 MPa. The estimated growth rate was similar for DCH with an a_w of 0.96 and 0.98 (trials 58–60, 63–64; Figure 5c,d). Nevertheless, it has to be considered that at pressure levels of 600–750 MPa, variability in the *L. monocytogenes* concentration was observed between sample replicates along the storage time (i.e., from

7 log cfu/g to not detected), making the growth-kinetic-parameter estimates less accurate (Table 2; Figure 5c,d). Some works have associated this variability in the *L. monocytogenes* counts after HPP to the different injury degrees and/or different recovery capacities of the *L. monocytogenes* cells [27].

4. Conclusions

This study provides the identification and quantification of the growth/no-growth limits and the subsequent behavior of *Salmonella* and *L. monocytogenes* in pressurized DCH. This information can be relevant for producers of DCH, which can take advantage of it to adopt process and/or product criteria that can be implemented in the Hazard Analysis and Critical Control Point (HACCP) plan to manage the safety of their products. In this respect, the data provided in this study highlight the importance of considering the storage temperature as a criterion throughout the entire food chain, since it is the main factor not only inhibiting the growth but also favoring the non-thermal inactivation of mesophilic *Salmonella* in pressurized DCH. In the case of the psychrotrophic *L. monocytogenes*, producers of DCH can consider the identified growth/no-growth threshold value of the a_w in the final product as a product criterion to be implemented in the HACCP with the aim of proving that their products can be classified as RTE foods unable to support the growth of *L. monocytogenes*.

The impact of the combination of factors, e.g., the intrinsic and extrinsic characteristics of the products on the behavior of pathogens after HPP is diverse, highlighting the need to evaluate the microbiological risk associated with DCHs on a case-by-case basis. In this framework, the quantified inactivation or growth kinetics of *Salmonella* and *L. monocytogenes* in pressurized DCHs with different intrinsic characteristics can help producers of DCH to detect scenarios of particular risk comprising HPP lethality and/or the survival and growth of pathogens during storage. Moreover, producers of DCH can take advantage of the data provided in this study to enhance the safety of their products by designing strategies, such as the application of corrective storage before DCH commercialization, to reach the performance criteria for *Salmonella* and *L. monocytogenes* and thereby to enhance the compliance with regulatory, customer, and internal requirements during the shelf-life of DCH.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app122412732/s1, Figure S1: Detailed description of the modeling procedure for calculating the gamma (γ_X) values. Table S1: Cardinal parameters of *Salmonella* and *L. monocytogenes* used to estimate the growth/no-growth boundary through the gamma concept in the present study. Table S2: Gamma factors (γ), interaction term between factors (ξ), and overall gamma factors (Γ) calculated for *Salmonella* and *L. monocytogenes* according to the extrinsic and intrinsic characteristics of dry-cured hams (DCH) used in this study. Refs. [15,22,23,28–30] are cited in the Supplementary Materials.

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Article 11

Modeling and designing a *Listeria monocytogenes* control strategy for dry-cured ham taking advantage of water activity and storage temperature

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Modeling and designing a *Listeria monocytogenes* control strategy for dry-cured ham taking advantage of water activity and storage temperature

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Abstract

Dry-cured ham is a shelf stable product that can be contaminated with *Listeria monocytogenes* due to post-processing operations, compromising the compliance of zero tolerance policies (e.g. US Listeria rule). The present study quantifies the behaviour of *L. monocytogenes* in sliced Spanish dry-cured ham of different water activity (a_w) during storage at different temperatures. Inactivation kinetics were estimated by fitting primary models to the experimental data. The effect of temperature and a_w on kinetic parameters was characterized through secondary polynomial models. *L. monocytogenes* viability decreased in all the assayed conditions, confirming that dry-cured ham is not only listeriostatic but listericidal. The fastest and highest reductions were observed at 25 °C, with 1 Log reduction after 6 and 9 days in Iberian and Serrano ham respectively. The work provides scientifically-based data and models to design a low-cost control measure based on a corrective storage as a post-lethality treatment to enhance the accomplishment of zero tolerance requirements.

Keywords

Listeria monocytogenes; RTE meat products; modeling; non-thermal inactivation; post-lethality treatment

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1 Introduction

Dry-cured ham is a raw ready-to-eat (RTE) meat product highly appreciated worldwide for its particular sensory characteristics. In 2017, the production reached the 299,000 tones in Spain, more than 15% being intended for export, which represents a 70% increase of the tones exported in 2012 (ANICE, 2019). The traditional EU markets have been mainly France, Germany, Portugal, Italy and the United Kingdom. Major emerging markets like Mexico, USA, Australia, South Korea, Chile, Japan, Argentina and New Zealand are foreseen of a great importance for the Spanish meat sector (ANICE, 2019). Dry-cured ham is considered a shelf-stable RTE product due to its low water activity (a_w) resulting from the salting and drying process of manufacture that renders a product with a high salt content up to 15% of the dry matter (Costa-Corredor, Serra, Arnau, & Gou, 2009; FSIS, 2010). Besides, the manufacturing process of dry-cured ham includes several steps, such as salting, post-salting, curing and drying/aging, with a duration depending on the type of dry-cured ham (from 7 months in the case of Serrano type, up to 18 to 48 months for Iberian type). The processing conditions have been proved to be lethal for *Listeria monocytogenes*, reducing the levels of the pathogen when inoculated in meat raw material by 4 Log units (Reynolds, Harrison, Rose-Morrow, & Lyon, 2001) in US type of dry-cured ham to 6 Log units in Spanish type dry-cured ham (Medina, 2017).

However, it has also been demonstrated that when marketed as convenient packaged formats (e.g. boneless blocks, diced, sliced), post-processing manipulation exposes the product to cross-contamination with pathogens, *L. monocytogenes* being of particular concern due to its ubiquitous nature and persistence in processing areas (Martín, Perich, Gómez, Yangüela, Rodríguez, Garriga, et al., 2014; Talon, Lebert, Lebert, Leroy, Garriga, Aymerich, et al., 2007). The contamination during post-processing operations is highly dependent on the production plant, with a prevalence reported between 3.6% and 18.4% (Prencipe, Rizzi, Acciari, Iannetti, Giovannini, Serraino, et al., 2012). The overall occurrence of *L. monocytogenes* in retail dry-cured ham varies from not detected (Cabedo, Picart-Barrot, & Teixidó-Canelles, 2008; Giovannini, Migliorati, Prencipe, Calderone, Zuccolo, & Cozzolino, 2007) to a prevalence of *ca.* 2% (Jemmi, Pak, & Salman, 2002; Prencipe, et al., 2012), 4% (Giovannini, Migliorati, Prencipe, Calderone, Zuccolo, & Cozzolino, 2007) and up to 12% (Uyttendaele, De Troy, & Debevere, 1999).

Food safety criteria regulations regarding *L. monocytogenes* in RTE products differ between countries. For EU member states, Regulation (EC) 2073/2005 establishes a maximum of 100 CFU/g of *L. monocytogenes* during the shelf-life of the product provided it is not intended for infants or special medical purposes or it does not favor the growth of the pathogen to more than 100 CFU/g at the end of shelf-life. This regulation states that RTE foods with a_w equal or below 0.92 automatically are considered to belong to the category of RTE food unable to support the growth of *L. monocytogenes* (European Commission, 2005). This a_w value is usually used by manufacturers as the acceptable limit for the commercial production of dry-cured ham. A similar tolerance approach is applied by Canadian regulation (Health Canada, 2011) and that of Australia and New Zealand (Australian Government, 2017). In contrast, in the US *Listeria* rule (FSIS, 2015), a zero-tolerance policy is imposed, which means that RTE products must not be released if they contain *L. monocytogenes* or have been in contact with a food contact surface contaminated with the pathogen. To meet this requirement, the establishment producing RTE foods exposed to *L. monocytogenes* contamination can apply control alternatives, based on antimicrobial agents or processes (AMA/P) to suppress pathogen growth and/or post-lethality treatments (PLT) to eliminate or reduce *L. monocytogenes* (FSIS, 2015).

Although, to the authors knowledge, no listeriosis case or outbreak has been associated with dry-cured ham, the pressure derived from zero-tolerance policies of the public health authorities of some countries as well as commercial demands, poses a challenge for the dry-cured meat industry due to the technical difficulties for the control and eradication of *L. monocytogenes*. To fulfil legal and/or commercial requirements, dry-cured ham producers should design risk minimization strategies to avoid sources of recontamination and/or apply validated PLT before commercial expedition. For dry-cured ham, thermal based post-lethality treatments are not suitable due to the negative impact on the organoleptic properties. Emerging non-thermal alternatives, such as high pressure processing have been proposed, though they

show limited effect due to the piezoprotection caused by the low a_w of the product (Bover-Cid, Belletti, Aymerich, & Garriga, 2015; Hereu, Bover-Cid, Garriga, & Aymerich, 2012) . Moreover, the economical investment needed to implement high pressure processing are not affordable for many producers. Therefore, feasible alternative strategies based on the physicochemical properties of the product itself should be investigated.

In this framework, the present study aimed to evaluate through a modeling approach the behaviour of *L. monocytogenes* in dry-cured ham, as a function of product a_w and storage temperature. The final objective was to design a feasible control measure contributing to ensure the accomplishment of zero-tolerance policies and commercial requirements. The study was carried out in two Spanish dry-cured ham types as the most typical and appreciated by consumer, Iberian ham and Serrano ham, showing differences in raw material (Iberian vs white pigs, respectively) and the process conditions, including length (up to 600 days vs 210 days, respectively) leading to end-products with different quality and prize (Lorido et al. 2015)

2 Material and methods

2.1 Product characteristics

Two different types of dry-cured ham were studied: Serrano and Iberian. Three batches for each type with different weight loss (high, medium, low), corresponding to a_w values of 0.87, 0.89 and 0.91 (Serrano type) and 0.85, 0.88 and 0.91 (Iberian type) in central sections of the ham piece, were used to study the impact of different values of a_w on the *L. monocytogenes* growth. Samples of hams were obtained directly from the producer, in vacuum-packed boneless blocks format and stored under refrigeration (<2 °C) until being used. Special attention was paid to obtain sections with the target a_w, which was measured at 25 °C using an Aqualab® equipment (Decagon Devices, Pullman, WA, USA).

2.2 L. monocytogenes strains and inoculum preparation

A cocktail of equal concentration of four *L. monocytogenes* strains with different genotype and serotype (Table 1), isolated from pork meat industrial environment (Medina, 2017; Ortiz, López, Villatoro, López, Carlos Davila, & Martínez-Suárez, 2010) was used . The strains were kindly provided by Dr. M. Medina (INIA, Spain). Stock cultures of each strain were kept at -80 °C in Brain Heart Infusion (BHI) broth (Beckton Dickinson, Sparks, Md., USA). A culture of each strain was separately grown in Tryptic Soy Broth with 0.6% Yeast Extract (TSBYE, Difco) following two consecutive incubation steps: firstly 18 h at 37 °C and secondly 4 days at 8 °C to obtain cold-adapted early stationary phase cultures according to the recommendations of the technical guidance document for conducting shelf-life studies on *Listeria monocytogenes* in RTE (EURL Lm, 2014). This physiological state (cold adaptation) mimics the chilled conditions usually found in clean rooms for production of RTE products (e.g. conveyor belts, slicing machines and packaging equipment).

2.3 Challenge test: sample preparation, inoculation and storage conditions

Boneless block hams (Serrano and Iberian, described in section 2.1) were aseptically sliced. Each slice (of *ca.* 20-30 g) was inoculated (1% v/w) with the 4-strain cocktail of *L. monocytogenes* described above to achieve *ca*. 10^6-10^7 CFU/g by properly diluting the culture in saline solution (0.85% NaCl and 0.1% Bacto Peptone (Beckton Dickinson)). The inoculum was spread on the dry-cured ham slice and left to absorb for 2 min under a laminar flow cabinet. The slices were overlaid cut in two and each part was individually vacuum packaged (in a EV-15 vacuum packer; Tecnotrip, Terrassa, Spain) in PA/PE bags (oxygen permeability of 50 cm^3/m^2/24 h and a low water vapor permeability of 2.8 g/m^2/24 h; Sistemvac, Estudi Graf S.A., Girona, Spain). Samples of each type of dry-cured ham were randomly distributed in 4 groups to be stored at 2, 8, 15 and 25 °C for a maximum of 6 months. These temperatures cover the reasonably foreseeable range for the storage and commercially display dry-cured ham, which has a maximum shelf-

life of 6 months under refrigeration. The a_w value of the samples was not significantly different after the inoculation. A total of 390 samples were prepared.

Table 4. *Listeria monocytogenes* strains used in this work[a].

Strain	Genotype	Serotype
EF 051005/3/A	S2	1/2a
EF 151105/2/A	S4-2	1/2b
EF 010207/24/A	S12-1	1/2c
EF 270406/1/A	S7-2	4b

[a]: strains were isolated from pork meat industrial environment (Medina, 2017; Ortiz, López, Villatoro, López, Carlos Davila, & Martínez-Suárez, 2010)

2.4 Monitoring L. monocytogenes behaviour

To monitor *L. monocytogenes* survival, samples from 24 experimental conditions (2 types of dry-cured ham, 3 a_w and 4 storage temperatures) were periodically analyzed to get a total of 12 to 19 data points distributed all along the storage period. This resulted in 201 and 189 samplings for Serrano and Iberian ham, respectively. Each sample was homogenized 1/10 in saline solution in a bag Blender Smasher® (bioMérieux, Marcy-l'Etoile, France) for 1 minute and 10-fold serially diluted in saline solution. *L. monocytogenes* was enumerated on Chromogenic Listeria Agar (CLA; Oxoid Ltd., Basingstoke, Hampshire, UK) and incubated at 37 °C for 48 h. For samples with expected concentration of *L. monocytogenes* below the quantification limit of 4 CFU/g (resulting from plating 4 ml of homogenate in a 14 cm diameter plate), the presence/absence of the pathogen was investigated by enrichment of 25-30 g-samples in 225 ml of TSBYE and incubated 48 h at 37 °C. After enrichment, the presence of *L. monocytogenes* was confirmed by plating on CLA (Sara Bover-Cid, Serra-Castelló, Dalgaard, Garriga, & Jofré, 2019). For modeling purposes, samples below the detection of plate count with positive after enrichment were assumed to be 1 cell in 30 g (i.e. -1.5 Log cfu/g). Negative results (i.e. not detected in 25-30g) were not recorded in any analyzed sample.

2.5 Primary model fitting

Four different inactivation primary models (Table 2), including the Weibull, Log-linear, Log-linear with tail and Log-linear with shoulder models (as described in Hereu, Dalgaard, Garriga, Aymerich, Bover-Cid, 2012) were used. For modeling purposes, to avoid small differences in initial concentrations, models were fitted to the *L. monocytogenes* inactivation data, expressed in terms of Log (N/N_0) (Martino & Marks, 2007) as a function of time (days) for each of the 24 combinations of conditions (type of ham, a_w and storage temperature). In addition, the Log N/N_0 at time zero (the initial inactivation) was fixed to 0 for parsimony purposes. All primary models were fitted using R with the nls2 and nls packages of R software (R Core Team, 2019).

Besides visual evaluation of the fitted curves, the standard error of the coefficients, the residual sum of squares (RSS) and the adjusted coefficient of determination (R^2_{adj}) were calculated as measures for goodness of fit. The primary model with a better goodness of fit, e.g. lower RSS and higher R^2_{adj} was chosen.

2.6 Secondary model fitting

Polynomial models were developed to quantify the effect of the independent variables (a_w and storage temperature) on the primary kinetic parameters. Different transformations, including square root, inverse, Ln and Log, of the primary kinetic parameters were assessed. Estimation of the model parameters was

carried out with R software (R Core Team, 2019) applying stepwise backward linear regression to obtain equations with only the significant parameters. The standard error of the coefficients, RSS and R^2_{adj} were calculated as measures for goodness of fit.

Besides the two-step modeling approach described above, the global one-step regression was applied for the fine tuning of the model parameters of *L. monocytogenes* inactivation on Serrano and Iberian type hams. For this, the secondary models for δ and p were integrated into the primary Weibull model and the combined model was fitted to the entire set of inactivation data points by one-step global non-lineal regression approach (Jewell, 2012; Martino & Marks, 2007).

The goodness of fit the one-step global models were assessed in terms of standard error of the coefficients, RSS and R^2_{adj} and by using graphs of observed and fitted values. The F-test was applied to assess the need of two different models for each product type compared to the suitability of a single model for both types of dry-cured ham.

Table 2. Primary inactivation models used to fit the *L. monocytogenes* inactivation data as a function of time.

Model	Equation[a]
Weibull	$Log(N/N_0) = -\left(\frac{t}{\delta}\right)^p$
Log-linear	$Log(N/N_0) = -\left(\frac{k_{max} \cdot t}{\ln(10)}\right)$
Log-linear with tail	$Log(N/N_0) = \text{Log}\left[\left(1 - 10^{Log(N_{res})}\right) \cdot e^{(-k_{max} \cdot t)} + 10^{Log(N_{res})}\right]$
Log-linear with shoulder	If $t \leq shoulder$; $Log(N/N_0) = 0$ If $t > shoulder$; $Log(N/N_0) = -\left(\frac{k_{max} \cdot t}{\ln(10)}\right) + Log\left(\frac{e^{(k_{max} \cdot shoulder)}}{1 + [e^{(k_{max} \cdot shoulder)} - 1] \cdot e^{(-k_{max} \cdot t)}}\right)$

[a] *Log (N/N₀)*: bacterial inactivation at specific time (*t*); Log N_{res}: inactivation tail (maximum inactivation); *t*: time (days); δ: time for the first Log reduction; *p*: shape of the inactivation curve; k_{max}: inactivation rate; *shoulder*: time before inactivation (initial resistance to stress).

2.7 Model predictive performance

Inactivation data recorded for *L. monocytogenes* on dry-cured Serrano and Iberian hams collected from scientific literature (Bover-Cid, Jofré, & Garriga, 2016; Hereu, Bover-Cid, Garriga, & Aymerich, 2012; Morales, Calzada, & Nuñez, 2006) were compared with predictions obtained by the models developed in the present study. To compare the observed and predicted inactivation during storage, the Acceptable Simulation Zone (ASZ) approach was used. Simulations were considered acceptable when at least 70% of the observed Log (N/N_0) values were inside the corresponding acceptable zone, e.g ± 0.5 (Møller, Ilg, Aabo, Christensen, Dalgaard, & Hansen, 2013).

3 Results and discussion

3.1. Description of the behaviour of L. monocytogenes on sliced dry-cured ham

The survival of *L. monocytogenes* under the 24 experimental conditions assayed is shown in Figure 1.The viability of *L. monocytogenes* was compromised in all the 24 conditions assayed, showing in most of the cases a significant reduction of the counts during the storage of sliced and vacuum packed dry-cured ham.

Therefore, the results indicated that under these conditions dry-cured ham is not only listeriostatic but listericidal. The magnitude of the lethal effect varied significantly according to the product characteristics and storage temperature. Thus, Iberian type ham favored an earlier and more pronounced inactivation of *L. monocytogenes*, compared with Serrano type, even if a_w was similar. The greater inactivation of *L. monocytogenes* in Iberian type can hardly be explained by the slightly lower pH (5.7 in Iberian versus 5.9 in Serrano), and probably other non-determined intrinsic factors of the product may have contributed to these differences. In both types of ham, the lower the a_w the higher the inactivation of the pathogen.

The impact of the temperature during storage of sliced dry-cured ham was also very noticeable. At refrigeration temperatures (2 and 8 °C) the listericidal effect of the product was limited, especially in higher a_w products (*ca*. only 1 Log reduction was achieved after 6 months of storage). On the other hand, at higher temperatures, especially at 25 °C, the inactivation was considerably more intense, achieving between 6 and 7 Log reductions of the level of the pathogen within 2 and 3 months of storage. Reynolds et al. (2001) also reported higher inactivation of *L. monocytogenes* during storage at room temperature of post-processing inoculated dry-cured ham.

The loss of viability of *L. monocytogenes* in dry-cured ham under the tested storage conditions can be explained by the metabolic exhaustion phenomenon associated with antimicrobial hurdles. The characteristics of the product, pH and mainly a_w of the ham did not allow the growth of the pathogen. In non-growth conditions of shelf-stable foods, the microorganisms tend to die, and die more rapidly when the conditions of shelf-stability approach the limits of growth, for example, as in this case, at room temperature (Leistner, 2000). These results point out that proper storage conditions of dry-cured ham would favor inactivation of *L. monocytogenes* contaminating the finished products before their release to retail, distribution, export, etc. Thus, dry-cured ham manufacturers can take advantage of this phenomenon as an opportunity to design a control measure into their production process, e.g. a validated post-lethality treatment, in order to minimize the risk of non-compliance of the zero-tolerance requirements.

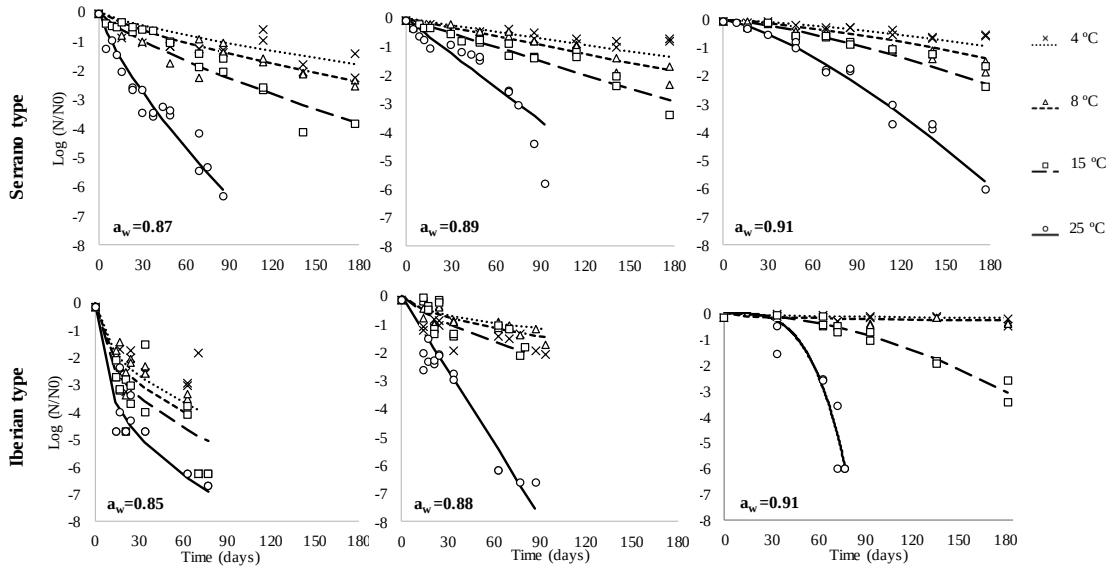

Figure 1. Behaviour of *L. monocytogenes* in Serrano and Iberian dry-cured hams with different a_w and stored at 2, 8, 15 or 25 °C. Symbols represent the observed pathogen inactivation, Log (N/N_0), and lines show the fit of the primary Weibull model.

3.2 Inactivation kinetics of L. monocytogenes on dry-cured ham. Primary modeling

Four primary inactivation models (Log-linear, Log-linear with tail, Log-linear with shoulder and Weibull) were fitted to inactivation data. The estimated kinetic parameters obtained using Log-linear based models

together with the goodness of fit are summarized in supplementary material (Table S1 for Serrano and Table S2 for Iberian dry-cured ham). The fitted kinetic parameter values and measures of goodness of fit obtained for the Weibull model are reported in Table 3. The graphical results of the Weibull model fit to inactivation of *L. monocytogenes* on sliced vacuum-packed dry-cured ham, according to the type of ham, a_w and storage temperature are shown in Figure 1. The Weibull model with two parameters (δ and p) allowed the fitting of different inactivation shapes through the p parameter and resulted in the best fit of the experimental data as indicated by the lower RSS and the higher R^2_{adj} values in comparison with the Log-linear based models. Therefore, the Weibull model was selected to describe the inactivation kinetics of *L. monocytogenes* on dry-cured ham.

The estimated δ parameter, e.g. the time needed for the first Log reduction, was systematically lower in Iberian than in Serrano ham, confirming that Iberian type favored an earlier inactivation of *L. monocytogenes*. In addition, the higher the storage temperature the lower the δ, pointing out that increasing up to room temperature favored the inactivation of the pathogen. On the other hand, the opposite effect was found for a_w, as the higher the a_w, the higher the δ. At refrigeration temperatures (e.g. 2 and 8 °C), especially for products with high a_w (>0.91), the δ showed values higher than 100 days, indicating that refrigeration slowed down the metabolic reactions preventing the metabolic exhaustion *L. monocytogenes* cells.

At the highest studied storage temperature (e.g. 25 °C) the shape of the inactivation curve (p) was highly dependent on the a_w of the product. In low a_w hams (0.85 and 0.87), *L. monocytogenes* inactivation showed a concave shape (p<1), indicating a higher inactivation at the beginning of the storage, and thus, the occurrence of a sort of tail of resistant cells. On the other hand, in products with the highest a_w (0.91), *L. monocytogenes* fate showed a convex shape (p>1), indicating lower inactivation at the beginning of the storage, and being in concordance with the highest time to the first 1 Log reduction (δ) found in higher a_w products compared to lower a_w products.

3.3 Secondary models for L. monocytogenes inactivation on dry-cured ham

Polynomial models were developed in order to quantify the impact of product a_w and storage temperature on the inactivation kinetic values obtained from the selected primary model fitting (e.g. the δ and p parameters of the Weibull model). Four different transformations were assessed, namely square root, inverse, Ln and Log.

The square root transformation of δ value was chosen for both products, Serrano and Iberian ham, as resulted with the best fit indicated by the higher R^2_{adj}. The δ parameter of both types of ham was dependent on product a_w and storage temperature. The F-test indicated that the equations for δ obtained for Serrano and Iberian hams were statistically different, thus a unique model for δ for both types of ham was not considered.

The best transformation of the p values was different depending on the type of ham. For Serrano ham, the inverse transformation of p values provided the best fit. It is noticeable that the transformed p values of *L. monocytogenes* in Serrano ham were statistically dependent on a_w but not on temperature, indicating the great effect of a_w on the shape of the inactivation curve. On the other hand, for Iberian ham the Log transformation fitted best the data and the resulting polynomial models indicated that p values were statistically dependent on temperature but also on the interaction between temperature and a_w.

The estimated parameters and the goodness of fit of the polynomial models developed for the inactivation of *L. monocytogenes* in dry-cured ham as a function of a_w and/or storage temperature are reported in Table 4.

Table 3. Estimated inactivation kinetic parameters resulting from fitting the primary Weibull model to the *L. monocytogenes* inactivation data obtained for dry-cured ham with different a_w and stored at different storage temperatures.

Product	Experimental conditions		Kinetic parameters			Goodness of fit[a]	
Dry-cured ham type	a_w	Temperature (°C)	δ (days)[b]	p[b]	n	RSS	R^2_{adj}
Serrano	0.87	2	34.9 ± 16.2	0.32 ± 0.13	16	0.123	0.677
		8	32.2 ± 10.2	0.48 ± 0.12	16	0.129	0.795
		15	47.5 ± 3.6	1.20 ± 0.08	19	0.062	0.947
		25	6.0 ± 1.1	0.65 ± 0.06	19	0.164	0.945
	0.89	2	>180	0.46 ± 0.08	16	0.013	0.860
		8	101.4 ± 5.2	1.28 ± 0.15	16	0.037	0.922
		15	64.2 ± 5.6	1.04 ± 0.12	19	0.075	0.900
		25	39.5 ± 3.4	1.93 ± 0.23	16	0.197	0.924
	0.91	2	>180	0.77 ± 0.16	16	0.010	0.798
		8	113.8 ± 3.9	1.14 ± 0.10	16	0.015	0.953
		15	105.3 ± 5.3	1.19 ± 0.14	16	0.035	0.911
		25	51.5 ± 3.4	1.41 ± 0.09	16	0.082	0.973
Iberian	0.85	2	2.0 ± 2.6	0.36 ± 0.16	16	12.675	0.542
		8	1.8 ± 1.3	0.30 ± 0.10	14	2.764	0.809
		15	2.0 ± 1.3	0.47 ± 0.10	18	14.073	0.769
		25	0.3 ± 0.2	0.33 ± 0.06	16	6.325	0.891
	0.88	2	15.8 ± 6.2	0.32 ± 0.10	16	1.291	0.745
		8	46.2 ± 6.5	0.46 ± 0.11	16	0.777	0.756
		15	38.3 ± 6.3	0.74 ± 0.20	17	2.690	0.559
		25	7.3 ± 1.2	0.79 ± 0.06	16	3.548	0.946
	0.91	2	-[c]	0.39 ± 0.31	16	0.363	0.249
		8	-[c]	0.32 ± 0.32	16	1.042	0.161
		15	100.8 ± 5.0	1.89 ± 0.18	16	0.777	0.954
		25	46.8 ± 5.2	3.55 ± 0.85	12	4.823	0.922

[a] n: number of inactivation data, Log (N/N_0), included for fitting, RSS: residual sum of squares; R^2_{adj}: adjusted coefficient of determination.
[b] Parameter estimate ± standard error.
[c] No inactivation was recorded. δ had an infinitive value.

In order to obtain refined model parameters, the equations obtained for the secondary models were combined with the selected primary model to use a single mathematical equation to fit the entire set of inactivation data though the one-step global fitting. The resulting readjusted values of the terms describing the inactivation of *L. monocytogenes* for the two types of dry-cured ham are shown in Table 4. The coefficients of equations of the global models clearly confirmed that different models were needed for Serrano and Iberian ham types because a combined model for the two types did not describe the experimental data appropriately. For each type of ham, statistical goodness of fit indices showed the one-step global models provided a better description of the inactivation data when compared to the classical two-step approach. This result was expected because the one-step global procedure fully considered the raw data, resulting in increased degrees of freedom and more accurate and robust parameter estimates (Jewell, 2012; Martino & Marks, 2007).

Table 4. Estimated coefficients of the polynomial models resulting from the fitting to values of the primary inactivation kinetics.

		Coefficients of the polynomial models[a]							Goodness of fit[b]		
	Serrano dry-cured ham	**a**	**b**	**c**	**d**	**e**	**f**	**g**	**P**	**RSS**	**R^2_{adj}**
Secondary polynomial models	$\sqrt{\delta} = a + b \cdot a_w + c \cdot a_w \cdot T$	-132.60 ± 34.32	163.19 ± 38.57	-0.33 ± 0.08	-	-	-	-	3	42.782	0.746
	$1/p = e + f \cdot a_w$	-	-	-	-	28.66 ± 10.84	-30.84 ± 12.18	-	2	4.748	0.330
Global model	$\mathrm{Log}(N/N_0) = \mathrm{Log}(N/N_0)_{initial} - \left(\frac{t}{(a + b \cdot a_w + c \cdot a_w \cdot T)^2}\right)^{\frac{1}{e+f \cdot a_w}}$	-88.52 ± 5.22	112.83 ± 5.84	-0.31 ± 0.01	-	13.93 ± 1.71	-14.51 ± 1.90	-	5	24.778	0.919
	Iberian dry-cured ham										
Secondary polynomial models	$\sqrt{\delta} = a + b \cdot a_w^2 + c \cdot T + d \cdot a_w \cdot T$	-90.99 ± 12.66	127.02 ± 16.31	3.96 ± 1.65	-4.66 ± 1.88	-	-	-	4	15.162	0.913
	$\mathrm{Log}\, p = e + f \cdot T + g \cdot a_w \cdot T$	-	-	-	-	-0.52 ± 0.08	-0.53 ± 0.10	0.63 ± 0.12	3	0.185	0.824
Global model	$\mathrm{Log}(N/N_0) = \mathrm{Log}(N/N_0)_{initial} - \left(\frac{t}{(a + b \cdot a_w^2 + c \cdot T + d \cdot a_w \cdot T)^2}\right)^{10^{(e+f \cdot T+g \cdot a_w \cdot T)}}$	-90.11 ± 7.44	127.42 ± 10.15	4.29 ± 0.65	-5.11 ± 0.76	-0.34 ± 0.07	-0.48 ± 0.05	0.56 ± 0.06	7	71.069	0.892

[a] Parameter estimates ± standard error.

[b] P: number of estimated parameters of the model; RSS: residual sum of squares; R^2_{adj}: adjusted coefficient of determination.

3.4 Evaluation of the developed models

After model formulation and selection based on its statistical performance to accurately describe the experimental dataset, it is important to evaluate the model predictive performance in real food systems with independently acquired data from similar food matrices. To this purpose, the Acceptable Simulation Zone (ASZ) approach was used to compare the 63 inactivation values obtained from scientific Articles dealing with *L. monocytogenes* in dry-cured ham with the respective predictions provided by the developed inactivation model (Table 5). Overall, the model tended to overestimate the inactivation of the pathogen by an average of 0.3 Log units, which can be considered satisfactory taken into account that it is a slight conservative (fail-safe) prediction. In addition, for Serrano ham, 72.9 % of the predictions were within the ASZ (Table 5), proving the good predictive performance of the developed models.

Due to the lack of independent data from Iberian ham, the evaluation of the developed *L. monocytogenes* inactivation model could not be properly conducted for this type of product. However, the few available data regarding Log (N/N_0) values of *L. monocytogenes* in Iberian hams collected from literature were all within the ASZ (Table 5).

3.5 Application of developed models

Within the alternatives recognized by the US Food Safety Inspection Service (FSIS) to control *L. monocytogenes* in RTE, the results of the present study constitute a scientific evidence that dry-cured ham can be considered an AMA/P, suppressing the growth of *L. monocytogenes* during the storage, thus making the product to fulfil the Alternative 2b requirements of the US Listeria rule (FSIS, 2015). It is worth to highlight that the listericidal effects observed in the present work during the storage of dry-cured ham could be exploited as PLT to achieve a level of control complying with Alternative 1 of US Listeria rule. For this, almost 1 Log reduction of *L. monocytogenes* before dry-cured ham is released to the market should be validated. The application of validated predictive models is an accepted option to validate PLT according to the FSIS (FSIS, 2014). In this framework, the predictive models developed in this study allow to set the time necessary to reduce 1 Log the level of *L. monocytogenes* at a given storage temperature for different types of dry-cured ham as a function of their a_w. To this aim, Figure 2 shows the 1-Log iso-reduction plots enabling the easy identification of time/temperature combinations suitable for a corrective storage (as the

PLT) for each type of dry-cured ham and a_w. In the lowest a_w products, the time required to achieve 1 Log reduction was of 9 and 6 days at 25 °C for Serrano and Iberian hams, respectively.

Table 5. Comparison of observed and predicted *L. monocytogenes* inactivation in Serrano and Iberian dry-cured hams.

Ref[a]	Dry-cured ham	a_w	Temperature (˚C)	Time (days)	Observed inactivation ($Log(N/N_0)$)	Predicted inactivation ($Log(N/N_0)$)	Observed-Predicted inactivation
[1]	Serrano	0.88	4	7	-0.71	-0.11	*-0.6*
		0.88	8	7	-0.59	-0.13	*-0.5*
		0.88	4	30	-1.24	-0.38	*-0.9*
		0.88	8	30	-1.38	-0.46	*-0.9*
		0.88	4	60	-1.35	-0.68	*-0.7*
		0.88	8	60	-1.25	-0.83	*-0.4*
[2]	Serrano	0.93	2	15	-0.05	0.00	*-0.1*
		0.93	2	15	0.01	0.00	*0.0*
		0.93	2	15	0.08	0.00	*0.1*
		0.93	2	15	-0.16	0.00	*-0.2*
		0.93	2	27	-0.04	-0.01	*0.0*
		0.93	2	27	-0.08	-0.01	*-0.1*
		0.93	2	41	-0.07	-0.02	*-0.1*
		0.93	2	43	-0.22	-0.02	*-0.2*
		0.93	2	55	-0.08	-0.03	*-0.1*
		0.93	2	70	-0.08	-0.06	*0.0*
		0.93	2	97	-0.25	-0.12	*-0.1*
		0.93	2	166	-0.22	-0.40	*0.2*
		0.93	2	166	-0.20	-0.40	*0.2*
		0.93	8	15	-0.28	0.00	*-0.3*
		0.93	8	15	-0.26	0.00	*-0.3*
		0.93	8	15	-0.14	0.00	*-0.1*
		0.93	8	15	-0.07	0.00	*-0.1*
		0.93	8	27	-0.16	-0.01	*-0.2*
		0.93	8	27	-0.27	-0.01	*-0.3*
		0.93	8	41	-0.26	-0.03	*-0.2*
		0.93	8	43	-0.12	-0.03	*-0.1*
		0.93	8	55	-0.37	-0.05	*-0.3*
		0.93	8	70	-0.15	-0.09	*-0.1*
		0.93	8	97	-0.67	-0.19	*-0.5*
		0.93	8	166	-1.20	-0.66	*-0.5*
		0.93	15	15	-0.23	-0.01	*-0.2*
		0.93	15	15	-0.46	-0.01	*-0.5*
		0.93	15	15	-0.23	-0.01	*-0.2*
		0.93	15	15	-0.20	-0.01	*-0.2*
		0.93	15	27	-0.46	-0.02	*-0.4*
		0.93	15	27	-0.23	-0.02	*-0.2*
		0.93	15	41	-0.15	-0.05	*-0.1*
		0.93	15	43	-0.26	-0.06	*-0.2*
		0.93	15	55	-0.57	-0.10	*-0.5*

Ref[a]	Dry-cured ham	a_w	Temperature (˚C)	Time (days)	Observed inactivation (Log(N/N$_0$))	Predicted inactivation (Log(N/N$_0$))	Observed-Predicted inactivation
		0.93	15	70	-0.88	-0.18	*-0.7*
		0.93	15	70	-0.92	-0.18	*-0.7*
		0.93	15	98	-1.36	-0.38	*-1.0*
		0.93	25	7	-0.18	0.00	*-0.2*
		0.93	25	7	-0.22	0.00	*-0.2*
		0.93	25	7	0.03	0.00	*0.0*
		0.93	25	7	-0.21	0.00	*-0.2*
		0.93	25	15	-0.29	-0.02	*-0.3*
		0.93	25	15	-0.17	-0.02	*-0.2*
		0.93	25	15	-0.21	-0.02	*-0.2*
		0.93	25	15	-0.25	-0.02	*-0.2*
		0.93	25	41	-0.92	-0.19	*-0.7*
		0.93	25	43	-0.81	-0.21	*-0.6*
		0.93	25	43	-0.94	-0.21	*-0.7*
		0.93	25	55	-0.98	-0.36	*-0.6*
[3]	Serrano	0.92	8	5	-0.52	0.00	*-0.5*
		0.92	8	13	-0.65	-0.01	*-0.6*
		0.92	8	32	-0.79	-0.06	*-0.7*
		0.92	8	61	-1.04	-0.18	*-0.9*
[3]	Iberian	0.88	8	5	-0.67	-0.27	*-0.4*
		0.88	8	13	-0.84	-0.46	*-0.4*
		0.88	8	32	-0.69	-0.79	*0.1*
		0.88	8	61	-0.7	-1.15	*0.5*

[a] References: *[1]* Morales et al. (2006); *[2]* Bover-Cid et al. (2016) *[3]* Hereu et al. (2012)

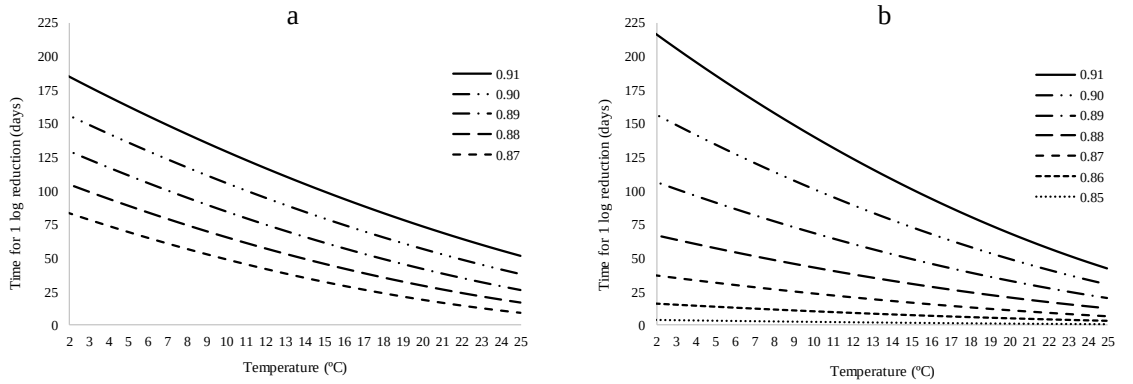

Figure 2. Predicted time for 1 Log reduction of *L. monocytogenes* according to the storage temperature in Serrano (a) and Iberian (b) hams with different a_w.

Considering that the estimated shelf-life of dry-cured ham is about 6 months, the application of such a short corrective storage time before product is released would be a feasible control measure as PLT, in form of a quarantine period, to reduce *L. monocytogenes* levels in products exposed to re-contamination after the drying process (e.g. during deboning, slicing, packaging) and thus, to ensure the accomplishment of the zero-tolerance policies, by operating under Alternative 1 of the Listeria rule (FSIS, 2015). This control

measure could also be helpful for companies within EU aiming to meet the commercial agreements of specific clients with zero tolerance requirements to their providers.

4 Conclusions

The physicochemical characteristics, mainly low a_w, make dry-cured ham not only listeriostatic but listericidal and thus, compromising the viability of *L. monocytogenes* depending on the product a_w and storage temperature.

In the framework of the design of risk minimization strategies, the quantified listericidal effect of dry-cured ham can be used to establish a corrective storage, a feasible low-cost control measure taking advantage of the product characteristics, as a PLT in products exposed to re-contamination after the drying process (e.g. during deboning, slicing, packaging). This measure could be implemented by the dry-cured ham producers to guarantee the fulfilment of restrictive legal and commercial requirements regarding *L. monocytogenes* derived from zero tolerance policies (such as the US Listeria rule).

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7 Supplementary data

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Article 12

Risk management tool to define a corrective storage to enhance *Salmonella* inactivation in dry fermented sausages

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Abstract

The resistance of *Salmonella* to the harsh conditions occurring in shelf-stable dry fermented sausages (DFS) pose a food safety challenge for producers. The present study aimed to model the behaviour of *Salmonella* in acid (with starter culture) and low-acid (without starter culture) DFS as a function of a_w and storage temperature in order to build a decision supporting tool supporting the design of a corrective storage strategy to enhance the safety of DFS. *Salmonella* spp. were inoculated in the raw meat batter at ca. 6 Log cfu/g with a cocktail of 3 strains (CTC1003, CTC1022 and CTC1754) just before mixing with the other ingredients and additives. After stuffing, sausages were fermented and ripened following industrial processing conditions. Different drying-times were applied to obtain three batches with different a_w (0.88, 0.90 and 0.93). Afterwards, DFS were stored at 4, 8, 15 and 25 °C for a maximum of three months and *Salmonella* spp. were periodically enumerated. The Weibull model was fitted to Log counts data to estimate inactivation kinetic parameters. The impact of temperature and a_w on the primary inactivation parameters was evaluated using a polynomial equation. The results of the challenge tests showed that *Salmonella* spp. levels decreased during storage at all the assayed conditions, from 0.8 Log (in low-acid DFS at 4 °C) up to 6.5 Log (in acid DFS at 25°C). The effect of both a_w and temperature was statistically significant. Delta (δ) parameter decreased by decreasing a_w and increasing temperature, while the shape (p) parameter ranged from above 1 (concave) at 10 °C to below 1 at 25 °C (convex). A common secondary model for the p parameter was obtained for each type of DFS, acid and low-acid, indicating that acidification during the production of DFS affected the time for the first Log reduction (δ) during the subsequent storage, but not the overall shape (p parameter) of the inactivation. The developed models covered representative of real conditions, such as *Salmonella* contamination in the raw materials and its adaptation to the harsh processing conditions. The good predictive performance shown when applying the models to independent data (i.e. up to 80% of the predictions within the 'Acceptable Simulation Zone' for acid sausages) makes them a suitable and reliable risk management tool to support manufacturers to assess and design a lethality treatment (i.e. corrective storage) to enhance the *Salmonella* inactivation in the product before DFS are released to the market.

Keywords

Pathogens; meat products; non-thermal inactivation; modelling; decision support tool; control measure

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1 Introduction

The production of dry fermented sausages (DFS) is one of the oldest forms of preserving meat (Ojha et al. 2015). As shelf-stable food, DFS do not support the growth of pathogenic microorganisms and refrigeration is not required to retain organoleptic acceptability. However, shelf-stability is not a guarantee of safety, which should be addressed in the production steps through a process not only inhibiting the growth but ensuring a sufficient reduction of the pathogens of concern that may be present in the raw materials.
The microbiological safety of DFS is mainly associated with the quality of raw materials and manufacturing practices, which determine the type and the initial levels of pathogenic microorganism potentially present (Barbuti & Parolari, 2002; Mutz et al., 2020) and the product formulation and the fermentation and drying conditions, which determine the time course of physicochemical characteristics changes during the DFS production process. Within this framework, pH (acidification due to the production of organic acids, mainly lactic acid) and water activity (a_w, reduction due to salting and drying) are the two intrinsic factors of high importance governing pathogen behaviour as part of the hurdle technology (Bonilauri et al., 2019; Leistner, 2000). Within the wide variety of DFS types, in Europe a differentiation between acid (usually northern) and low-acid (usually from Mediterranean area) DFS (Demeyer et al., 2000; Lebert et al., 2007) has been described. In acid non-thermally treated DFS and especially in mildly fermented low-acid (usually pH≥5.3) traditional DFS, typical from the Mediterranean region such as fuet, a low diameter DFS typical from Catalonia (Aymerich et al., 2003; Martin et al., 2011), the pathogen-controlling efficacy could be diminished and the safety of the product compromised (Jofré et al., 2009).
Salmonella is one of the most relevant pathogens in DFS due to its ability to survive acid and low a_w conditions (Mutz et al., 2020). Although a decrease of *Salmonella* loads during the DFS production process is usually reported, it was also shown to survive certain processes (Gunvig et al., 2016; Jofré et al., 2009; Martin et al., 2011; Skandamis & Nychas, 2007). DFS contaminated with *Salmonella* has been epidemiologically linked to several salmonellosis outbreaks. From 2016 to 2020, up to eight notifications were recorded in the EU Rapid Alert System For Food and Feed portal (RASFF, https://webgate.ec.europa.eu/rasff-window/portal/) about outbreaks in France, Sweeden, Denmark related with the presence of *Salmonella* in DFS from France, Spain, Italy and Poland (notification references 2020.5038; 2020.3378; 2018.1111; 2018.0246; 2017.1846; 2017.1511; 2016.1340; 2016.0492). These notifications highlight the ability of *Salmonella* to survive during the DFS production process and storage due to its resistance to acidity and low a_w conditions (Mutz et al., 2020; Tiganitas et al. 2009), posing an important challenge for food business operators to accomplish with the zero-tolerance policy for *Salmonella* (no detection in 25 g of n=5 analysed units per verified lot) required by current European food safety microbiological criteria regulation (European Commission, 2005). Therefore, the development of strategies based on post-processing treatments can be useful. For instance, non-thermal technologies such as high pressure processing of DFS has been studied (Bonillauri et al, 2019; Jofré et al., 2009; Porto-Fett et al., 2010) though the low a_w usually found in DFS exerts piezoprotection effect against *Salmonella* inactivation and reduces its efficacy (Bonilauri et al., 2019; Bover-Cid et al., 2012; Bover-Cid et al., 2017). Moreover, the investment cost of this technology is not always affordable by food producers. In this regard, strategies based on the enhancement of the hurdle technology, making the most of the physicochemical characteristics of DFS can be developed. For instance, few studies have proposed the implementation of a corrective storage period after the manufacturing process to enhance the reduction of verotoxigenic *Escherichia coli* in DFS (Hansen et al., 2011) and *Listeria monocytogenes* in dry-cured ham (Serra-Castelló et al., 2020), in both cases developing decision support tools for a proper implementation of such control measure. Hwang et al. (2009) modelled the survival of *Salmonella* during the storage of soudjouk-style fermented sausages, an acid type (pH <5.2) sausage made of beef, which does not cover the conditions of the small-diameter acid (pH<5.3) and low-acid (pH≥5.3) traditional European pork DFS.
In this framework, the present study aimed to evaluate the behaviour of *Salmonella,* inoculated in the raw materials before stuffing, during the storage of low-acid and acid DFS. The behaviour of *Salmonella* was tackled through a modelling approach, which quantified the pathogen inactivation as a function of the

product a_w and storage temperature. The final objective was to provide a risk management tool assisting the design of a feasible and cost-effective control measure contributing to ensuring the accomplishment of zero-tolerance policies and commercial requirements.

2 Material and methods

2.1 Salmonella strains

A cocktail of three strains of *Salmonella enterica* from IRTA-Food Safety Program`s collection isolated from pork meat products and belonging to different serotypes, i.e. CTC1003 (London), CTC1022 (Derby) and CTC1754 (Rissen), was used in the present study. Inoculum cultures were prepared by growing each strain independently in Brain Heart Infusion (BHI) broth (Beckton Dickinson, Sparks, Md., USA) at 37 °C for 7 h and subsequently sub-cultured again at the same temperature for 18 h (i.e. till the stationary phase of growth was reached). Final cultures were preserved frozen at −80 °C in the growth medium supplemented with 20% glycerol until being used (Hereu et al., 2012).

2.2 Preparation, inoculation, processing and storage of dry fermented sausages

Meat batter was prepared by mixing minced lean pork meat and fat (4:1). Following the mixing, the meat batter was inoculated with a cocktail of the three *Salmonella* strains (0.3% v/w) prepared by mixing equal number of cells for each strain using frozen cultures prepared as described in section 2.1 and diluted in water to achieve a final concentration of *ca*. 6 Log cfu/g. The meat batter was mixed for 1 min in the mixing machine (Mix-35P, Tecnotrip, Spain) in order to homogenize the inoculum in the batter before adding the following ingredients and additives (in g/kg): water, 30; NaCl, 18; dextrose, 5; black pepper, 2; sodium ascorbate, 0.5; $NaNO_2$, 0.1; KNO_3, 0.1. Finally, in low-acid DFS batches no starter culture was added, while a mixture with the starter cultures *Lactobacillus sakei*, *Pediococcus pentosaceus* and *Staphylococcus xylosus* (Aymerich et al., 2003; Marcos et al., 2007) was added to produce acid DFS batches. After addition of the cultures, the meat batter was mixed for an additional 1 min.

The inoculated meat batters were stuffed in 36-38 mm diameter natural pork casings using a stuffing machine (H15, Tecnotrip, Spain) and sausages of ca. 25 cm in length were elaborated. After, sausages were dipped into a solution of *Penicillium candidum* and *P. nalgiovensis* spores (Danisco, France). Sausages were let to dry at room temperature (18 - 20 °C) for the dripping/drying of the casings and subsequently hung in a Versatile Environmental Test Chamber MLR-350 H (Sanyo Electric Co., Ltd. Japan) adapted with an Hygrotest 600 PHT-20/120 transmitter (Testo) for the fermentation and drying processes. Sausages were fermented for 1 day at 22 °C with 85-86 % of relative humidity (RH). Afterwards, during the drying process, the RH conditions were set up to gradually decrease RH from 85 to 65 % and increase temperature from 13 to 18 °C. With the aim to obtain sausages with different a_w (0.88, 0.90 and 0.93), the duration of the drying processes was 20, 19 and 10-11 days, respectively.

A total of six batches of DFS were obtained, combining different a_w (0.88, 0.90 and 0.93) and pH (≥5.6 and ≤5.1 for low-acid and acid DFS, respectively). For details on the physicochemical and microbiological analysis, including *Salmonella* counts, of the products during the processing see Supplementary Table 1. The obtained DFS were stored in perforated plastic bags and randomly distributed in four groups to be stored at foreseeable storage temperatures (i.e. 4 °C, 10 °C, 15 °C and 25 °C) for a maximum of three months.

2.3 Microbiological analysis during the ripening processes and the subsequent storage

The levels of *Salmonella* were monitored by sampling along the production process (15 samples type of DFS and final a_w value) and storage period (with a total of 10-30 samples depending on the storage temperature).

After aseptically removing the casing, 25 g of sausage were homogenized ten-fold in saline solution (0.85 % NaCl and 0.1 % Bacto Peptone (Beckton Dickinson)) in a bag Blender Smasher® (bioMérieux, Marcy-l'Etoile, France) for 1 min and 10-fold serially diluted in saline solution. *Salmonella* was enumerated on the selective and differential chromogenic *Salmonella* agar (CHROMagar™ *Salmonella* Plus; Scharlab, S.L., Sentmenat, Spain) after incubation at 37 °C for 24 - 48 h. Samples with expected *Salmonella* concentration below the quantification limit (4 cfu/g) were enriched in Rappaport-Vassiliadis (RV) broth (Oxoid Ltd., Basingstoke, Hampshire, UK) and incubated at 41 °C for 24 h. After enrichment, the presence of *Salmonella* was checked by plating on the chromogenic *Salmonella* agar. The absence of *Salmonella* in non-inoculated meat batter was confirmed in all the batches.
Levels of Lactic Acid Bacteria (LAB) were determined during the production process of the sausages in MRS (de Man, Rogosa and Sharpe) agar plates (Merck, Darmstadt, Germany), which were incubated at 30 °C for 72 h under anaerobiosis using sealed jars with an AnaeroGen sachet (Oxoid Ltd.).

2.4 Primary modelling of the Salmonella behaviour during storage

For each combination of the conditions (acidity/a_w/storage temperature), the primary Weibull model (Eq. 1) was fitted to the *Salmonella* survival data (Log N) as a function of the storage time using the nls2 and nls packages of R (R Core Team, 2019).

$$\mathrm{Log}(N) = \mathrm{Log}(N_0) - \left(\frac{t}{\delta}\right)^p \qquad \text{Eq. 1}$$

Where $Log(N)$ is the *Salmonella* concentration at given time, $Log(N_0)$ is the average value of the initial *Salmonella* concentration of three replicates at time zero of the storage period (i.e. end of drying), δ is the time (days) required for the first Log reduction of *Salmonella*, p is a dimensionless parameter describing the shape of the inactivation curve (i.e. $p < 1$ concave; $p = 1$ linear and $p > 1$ convex) and t is the storage time (days).
The goodness-of-fit of the developed models was assessed by standard error of the parameter estimates, residual sum of squares (RSS), root mean squared errors (RMSE).

2.5 Secondary model fitting

Polynomial models were developed to quantitatively characterize the effect of a_w and storage temperature on the kinetic inactivation parameters (δ and p) resulting from the primary modelling (Table 2).
Following the parsimony principle, the fit of the polynomial models to the kinetic inactivation parameters, transformations (including square root, inverse, Ln and Log) were assessed throughout the application of stepwise regression to obtain equations with only the significant parameters. Estimation of model parameters and the associated standard errors was conducted with the *nls* and *lm* function of the *nls2* and *stats* packages of the R software (R Core Team, 2019).
Besides the classical two-step modelling approach described above, the one-step or global modelling approach (Jewell, 2012; Martino & Marks, 2007) was applied, i.e. secondary polynomial models for the inactivation parameters (δ and p) were integrated into the Weibull primary model equation. The goodness-of-fit of the developed models was assessed by the standard error of the parameter estimates, RSS and the RMSE. The F-test (Eq. 2) was applied to assess the need of two different models for low-acid and acid DFS (Zwietering et al., 1990).

$$F = \frac{\left(RSS_{NH} - RSS_{AH}\right) / \left(df_{NH} - df_{AH}\right)}{RSS_{AH} - df_{AH}} \qquad \text{Eq. 2}$$

Where RSS_{NH} and df_{NH} were the Residual Sum of Squares and the degrees of freedom (number of points-number of parameters of the model) respectively, of the global model common for both types of DFS (null hypothesis) and RSS_{AH} and df_{AH} were the Residual Sum of Squares and the degrees of freedom respectively, of the global model with specific parameter coefficients for each type of DFS (alternative hyphotesis).
The effect of the environmental conditions on the shape inactivation curve of *Salmonella* was assessed with the comparison of two global models: i) a global model with a polynomial model for describing the effect of temperature and a_w on the *p* parameter and ii) a global model with a fixed *p* value independent of the environmental conditions. The comparison was assessed using the F-test (Eq. 2), where RSS_{NH} and df_{NH} were the Residual Sum of Squares and the degrees of freedom (number of points-number of parameters of the model) respectively, of the constrained model (global model with fixed p value; null hypothesis) and RSS_{AH} and df_{AH} are the Residual Sum of Squares and the degrees of freedom respectively, of the global model with a polynomial model describing the effect of temperature and a_w on the *p* parameter (alternative hypothesis).

2.6 Evaluation of the model performance

Predictions obtained by the models developed were compared with totally independent data obtained by the Technical University of Denmark (DTU) about *Salmonella* behaviour in acid and low-acid fermented sausages during storage after being fermented and dried under the conditions detailed in Gunvig et al. (2016). The data are included in Supplementary Tables 2 and 3. The Acceptable Simulation Zone (ASZ) approach was used to compare the predicted and observed *Salmonella* reduction during the storage of the DFS. Due to the scattering of the observed data, simulations were considered acceptable when at least 70% of the observed Log N values were inside the acceptable zone of ± 1 Log (Møller et al., 2016).

3. Results and Discussion

3.1 Salmonella, lactic acid bacteria and pH during the fermentation and drying processes

In low-acid DFS without starter culture, a slight increase of *Salmonella* was observed during the first days of the process, followed by a slight decrease, with a total reduction of less than 1 Log unit. In this type of sausages, LAB took at least 7 days to reach the stationary phase (i.e. 8 Log cfu/g) and pH did not decrease below 5.3 (Supplementary Table 1). In acid DFS, LAB reached the stationary phase levels in just 1 day and the pH decreased down to 4.6-4.8. The highest reduction of *Salmonella* levels, 2.5-2.7 Log units, was recorded for those processes leading to acid DFS with the lowest a_w (0.88 and 0.90) highlighting the role of acidification on the loss of viability of *Salmonella*. On the other hand, in DFS with a higher a_w (0.93) *Salmonella* counts only decreased by ca. 1 Log, being statistically similar (p-value > 0.05) to the pathogen inactivation observed in low-acid DFS with the same a_w. As a result of the different behaviour of *Salmonella* occurring during the production of the different types of sausages, the levels and the physiological status of the pathogen in the end-product (at the beginning of the storage) were not equal in all the conditions studied and this might have influenced the subsequent behaviour during the storage at different temperatures (section 3.2). It has been described that to survive stresses intrinsically associated with fermentation and drying, *Salmonella* develops complex mechanisms of stress adaptation increasing its tolerance and survival against harsh environmental conditions, thus affecting the behaviour during the subsequent storage of DFS (Mutz et al., 2020). The behaviour of *Salmonella* during the storage of DFS has been frequently investigated inoculating the pathogens on slices of the end product (Calicioglu et al., 2002), Dalzini et al., 2014; Porto-Fett et al., 2008). However, this approach does not represent the actual contamination event, as *Salmonella* comes from contaminated raw materials (Barbuti & Parolari, 2002) with a relevant prevalence in fresh pig meat used for DFS manufacture (up to 23.7%, Martin et al., 2011).

3.2 Salmonella behaviour during storage of low-acid and acid sausages

Figure 1 shows the survival of *Salmonella* during storage in the 24 combination of conditions assayed. Results indicated that under the evaluated conditions, both low-acid and acid DFS were not only bacteriostatic but also bactericidal against *Salmonella*.

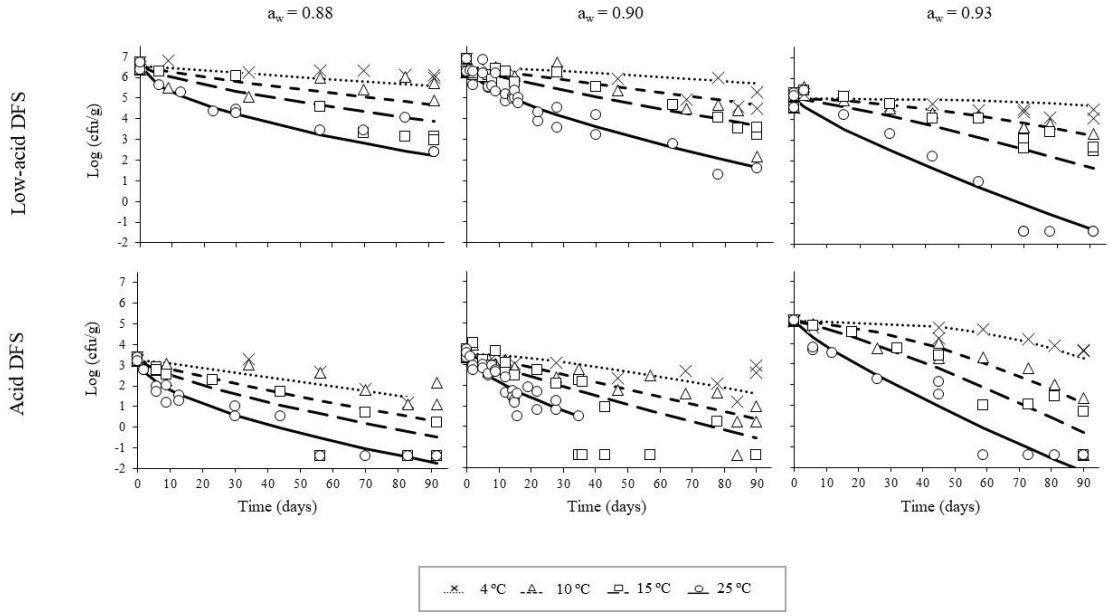

Figure 1. Behaviour of *Salmonella* in low-acid and acid dry fermented sausages (DFS) with different a_w and stored at 4, 10, 15 and 25 °C. Symbols represent the observed pathogen counts in Log cfu/g (n=3) and lines show the fit of the global model shown in Table 2.

However, different extent of *Salmonella* inactivation was observed depending on the acidity and the a_w of the DFS as well as the storage temperature. Specifically, at the storage time of ca. 60 d, a reduction of 3.0 Logs in the *Salmonella* level was observed in low-acid DFS with a a_w of 0.88 and stored at 25 °C. At the same storage time and temperature, higher reductions of *Salmonella* were observed for the same type of DFS with higher a_w, 0.90 (3.7 Log) and 0.93 (4.0 Log), indicating that *Salmonella* could have adquired higher resistance during the manufacture of DFS with lower a_w that those showing higher a_w at the end of the drying. These findings are in agreement with those found by Farkos et al. (2013) dealing with low moisture foods inoculated with dried cells of *Salmonella*, showing an increased survival capacity with decreasing a_w of the matrix. In the work performed with *L. monocytogenes* inoculated in slices of dry-cured ham, the lower a_w the higher the inactivation (Serra-Castelló et al. 2020). In that case, however, *L. monocytogenes* was exposed to the product characteristics and storage conditions after the manufacturing, thus without previous adaptation, which can be the reason for the different impact of the a_w. In acid DFS, these reductions were enhanced by ca. 2 Logs, showing the relevance of the acidity of the product in promoting the pathogen inactivation. The effect of storage temperature was also very remarkable since no relevant *Salmonella* reduction (< 1 Log) was found after 60 d of storage at 4 °C in any of the products assessed. In summary, higher reduction of *Salmonella* was recorded in DFS with higher a_w and stored at higher temperatures and this inactivation was enhanced in acid DFS. These results highlight the importance of the product intrinsic factors (a_w and pH) and its combination with the storage temperature. In this regard, the observed bactericidal effect could be related with the metabolic exhaustion phenomenon associated with the combination of antimicrobial hurdles in agreement with the principles of the hurdle technology developed by Leistner (2000). Accordingly, in shelf-stable products with physicochemical characteristics not supporting the growth of microorganisms the viability of bacterial cells is compromised because they completely use up their energy trying to repair homeostasis mechanisms, causing a die-off of the microorganisms along the storage. The inactivation rate is known to be higher when the temperature

increases towards the optimal growth for the microorganisms as well as when some of the other physicochemical characteristics (pH or a_w) approach limits of the microbial growth (Leistner, 2000; Serra-Castelló et al., 2020). In the present study, room temperature storage, acidity and high a_w (0.93, i.e. the minimum a_w for *Salmonella* growth when other factors are optimal (ICMSF, 1996) of the DFS, would be conditions favouring metabolic exhaustion.
Overall, results indicated that the storage of low-acid and acid DFS at selected temperature conditions (e.g. 25 °C) would favour the inactivation of *Salmonella* cells, even adapted to the stress of fermentation and drying conditions. Therefore, sausage manufacturers can design a control measure into their manufacturing operations based on this phenomenon to minimize the risk of non-compliance with the *Salmonella* zero-tolerance policy.

3.3 Primary modelling of Salmonella behaviour during storage

The Weibull model (Eq. 1) was found to be appropriate to describe *Salmonella* reduction (inactivation) during the storage (Table 1), as also reported in other low-moisture foods (Santillana-Farakos, 2013), although the fit of the model was poor for low-acid DFS stored at low temperature (4°C) conditions due to the lack of inactivation within the time frame of the present experiment (3 months). This was the reason for the associated high standard errors of the Weibull parameters estimated for this particular case.

At the three evaluated levels of a_w, higher values of the δ parameter of the Weibull model, i.e. the time for the first Log reduction of *Salmonella*, were obtained in low-acid DFS compared with acid DFS with the same a_w and stored at the same temperature (Table 1), quantifying the enhanced *Salmonella* inactivation in acid DFS with reductions in δ of up to 2.4-fold in the driest DFS. Moreover, in both products, δ was increased with increasing a_w and decreasing storage temperature, indicating the enhancement of the *Salmonella* lethality due to the low a_w and high storage temperature (up to 25 °C). For example, in low-acid DFS with the lowest a_w (0.88), a ca. one week of storage at 25 °C would be enough to decrease *Salmonella* counts by 1 Log, but little or no microbiologically relevant *Salmonella* inactivation would be expected after 90 d at 4 °C.

These results were also supported by the p parameter of the Weibull model, that described different inactivation shape curves depending on the a_w of the product and storage temperature. At 25 °C, p values tended to be below 1 in most of the conditions, indicating a higher inactivation of the pathogen at the beginning of the storage followed by a slow down of the rate of inactivation of *Salmonella*. On the other hand, at lower storage temperatures, p values tended to be higher than 1 in most of the cases, corresponding to a convex curve (shoulder shape), indicating lower inactivation at the beginning of the storage, probably due to a slow down in the metabolism of *Salmonella* at temperatures close or below its minimum growth temperature, described to be below 7 °C for most serotypes (ICMSF 1996).

3.4 Secondary and global modelling

The Log transformation for δ parameter and the inverse transformation for p parameter were chosen for both products, i.e. low-acid and acid DFS, as they gave the best fit (Table 2). The polynomial models developed indicated that δ and p parameters were linearly dependent on a_w and storage temperature. In addition, the quadratic term found for a_w in the polynomial model for p, described the great effect of a_w on the shape of the *Salmonella* inactivation curve. Refined model parameters (Table 2) were obtained through the one-step (global) approach, integrating of the secondary polynomials developed for the inactivation parameters in the Weibull primary model equation and the re-fitting of this combined model to the entire set of 350 data points of *Salmonella* in both types of DFS. Interestingly, the F-test indicated that equations obtained for δ parameters of both products were statistically different but not the ones describing the p parameter, thus, a unique model for the p parameter was considered for both low-acid and acid DFS.

Despite not being significantly influenced by the type of product, *p* parameter showed to be affected by the environmental conditions, i.e. storage temperature and a_w.

Table 1. Estimated inactivation kinetic parameters resulting from fitting the primary Weibull model to the *Salmonella* counts obtained for low-acid and acid dry fermented sausages (DFS) with different physicochemical characteristics and stored at different temperatures.

Product	Experimental conditions		Kinetic parameters			Goodness of fit[d]		
DFS	a_w (pH)[a]	T[b] ˚C	$LogN_0$[c] Log cfu/g	δ[d] days	p[d]	n[e]	RSS	RMSE
Low-acid	0.878 ± 0.002	4	6.53	156.11 ± 73.75	1.47 ± 1.05	10	0.220	0.166
	(5.68 ± 0.11)	10	6.53	130.00 ± 187.00	2.05 ± 6.63	10	0.227	0.168
		15	6.53	32.25 ± 5.14	1.25 ± 0.21	10	0.721	0.300
		25	6.53	6.81 ± 2.77	0.51 ± 0.09	13	2.178	0.445
	0.889 ± 0.001	4	6.47	64.85 ± 8.98	1.41 ± 0.67	12	1.690	0.411
	(5.60 ± 0.07)	10	6.47	49.16 ± 7.25	1.45 ± 0.47	12	1.306	0.362
		15	6.47	39.09 ± 4.34	1.34 ± 0.20	17	1.201	0.283
		25	6.47	8.93 ± 1.12	0.71 ± 0.05	30	4.879	0.417
	0.932 ± 0.000	4	5.02	105.67 ± 19.47	1.22 ± 0.56	13	0.585	0.231
	(5.64 ± 0.03)	10	5.02	59.47 ± 5.34	1.27 ± 0.36	12	0.722	0.269
		15	5.02	48.63 ± 5.88	1.48 ± 0.34	13	1.394	0.356
		25	5.02	13.82 ± 3.78	1.03 ± 0.17	13	4.942	0.670
Acid	0.883 ± 0.002	4	3.27	64.44 ± 6.10	3.01 ± 1.30	10	0.993	0.407
	(4.83 ± 0.15)	10	3.27	59.73 ± 9.05	1.43 ± 0.58	11	1.317	0.383
		15	3.27	15.29 ± 7.30	0.80 ± 0.24	13	7.030	0.799
		25	3.27	4.20 ± 0.97	0.51 ± 0.05	19	2.539	0.386
	0.903 ± 0.002	4	3.54	53.40 ± 17.81	0.50 ± 0.31	12	2.119	0.460
	(5.06 ± 0.03)	10	3.54	35.23 ± 7.82	1.28 ± 0.34	18	6.629	0.644
		15	3.54	9.91 ± 3.73	0.76 ± 0.16	24	27.077	1.109
		25	3.54	6.21 ± 1.17	0.67 ± 0.10	27	5.228	0.457
	0.930 ± 0.002	4	5.09	64.54 ± 16.66	0.48 ± 0.28	12	1.213	0.348
	(4.70 ± 0.05)	10	5.09	42.30 ± 8.06	2.04 ± 0.57	12	6.635	0.815
		15	5.09	26.45 ± 5.38	1.35 ± 0.25	13	4.622	0.648
		25	5.09	5.38 ± 2.08	0.67 ± 0.10	14	5.472	0.676

[a] a_w and pH of the DFS at the beginning of the storage ± standard deviation .
[b] storage temperature.
[c] $LogN_0$ is the average value of the initial *Salmonella* counts of three replicates at the beginning of the storage.
[d] Parameter estimates ± standard error.
[e] n: number of count data, i.e. Log (N), included for fitting. RSS: residual sum of squares; RMSE: root mean of squared errors.

The F-test (Eq. 2) statistical comparison between the global model with a polynomial model for the *p* parameter and the global model with a fixed *p* parameter, resulted in a high F value (121.09) showing that the constrained model (model with fixed *p*) could not explain the same variance as the complex model. Thus, results suggested that the effect of temperature and a_w has to be considered when characterizing the shape of the inactivation curve of *Salmonella* in fermented sausages.

Table 2. Estimated coefficients of the global model resulting from the fitting to values of the primary and secondary inactivation kinetics of *Salmonella* in dry fermented sausages.

		Sausage type	Coefficients of the polynomial models[a]						Goodness of fit[b]			
			a	b	c	d	e	f	n	P	RMSE	R^2_{adj}
Secondary modelling	$Log\ (\delta) = a + b \cdot T + c \cdot a_w$	Low-acid	2.03 ± 2.29	-0.05 ± 0.01	0.29 ± 2.53	-	-	-	12	3	0.18	0.831
	$1/p = a + b \cdot T + c \cdot a_w{}^2$		3.71 ± 3.09	0.05 ± 0.01	-4.00 ± 3.42	-	-	-	12	3	0.24	0.742
	$Log\ (\delta) = a + b \cdot T + c \cdot a_w$	Acid	0.92 ± 1.89	-0.05 ± 0.01	1.26 ± 2.09	-	-	-	12	3	0.15	0.887
	$1/p = a + b \cdot T + c \cdot a_w{}^2$		1.47 ± 3.15	0.05 ± 0.01	-1.40 ± 3.48	-	-	-	12	3	0.16	0.915
Global modelling	$\mathrm{Log}(N) = \mathrm{Log}(N_0) - \left(\frac{t}{10^{(a+b\cdot T+c\cdot a_w)}}\right)^{\frac{1}{d+e\cdot T+f\cdot(a_w{}^2)}}$	Low-acid	-0.40 ± 1.05	-0.06 ± 0.00	2.95 ± 1.15	5.54 ± 0.92	0.04 ± 0.00	-6.15 ± 1.09	350	9	1.05	0.882
		Acid	1.30 ± 1.03	-0.05 ± 0.00	3.59 ± 1.12							

[a] Parameter estimates ± standard error

[b] n: number of *Salmonella* counts (Log N). δ (days) or p values included for fitting; P: number of estimated parameters of the model; RMSE: root mean of squared errors; R^2_{adj}: adjusted coefficient of determination.

The inoculation of *Salmonella* in the raw materials of different types of sausages lead to different levels in the final product, i.e. at the beginning of storage. Despite this could be a drawback as different initial levels could affect the characterization of the behaviour of the pathogen, this is especially relevant when dealing with low inoculum levels, where the variability in the counts together with being in a region close to the plate count detection limit highly affect the shape of the inactivation/growth curves (Mataragas et al., 2015). However, in the present study the levels of *Salmonella* recorded at the end of the drying process (i.e. at the beginning of the storage period assessed), were high enough to allow a proper characterization of the shape of the inactivation curve of the pathogen during the storage time. At the same time, data covered the impact that the sequential exposure of *Salmonella* to stresses during the DFS manufacturing processes (acidification and drying) could have on the subsequent inactivation during the storage, which should not be covered if *Salmonella* had been inoculated in the end product without being exposed to the fermentation and drying. Tiganitas (2009) highlighted the impact of the order in the application of hurdles, showing that the lethality due to acid and osmotic stresses was higher when the stresses were applied sequentially compared to their simultaneous application. Our results would indicate that fermentation (acidification due to organic acids produced by LAB) would increase *Salmonella* sensitivity during storage. The impact of drying (low a_w) was different at the beginning of the storage in comparison with the later stages of the study. In this respect, for the δ parameter, the lower the a_w the shorter the time for the first log reduction, thus a lower a_w favored the early inactivation. However, when taking into account the long term data, considering the whole inactivation curve (p parameter), the results indicate that lower a_w resulted in lower total inactivation, indicating that the low a_w favored the occurrence of a tail of resistant cells.

Therefore, the model was built considering representative of foreseeable industrial conditions leading to different *Salmonella* levels and physiological states as a result of the different resistance and adaptation of the pathogen to the process conditions. The model will provide useful information to manufacturers producing different types of DFS to assess the feasibility of applying a short storage period prior to their release to the market, taking the advantage of the non-thermal inactivation effects of the product on *Salmonella*.

3.5 Effect of acidity on Salmonella inactivation during storage

Results from the secondary and global modelling (section 3.4) indicated that the level of acidification in DFS affected the time for the first Log reduction of *Salmonella* but not the inactivation curve shape. Therefore, contrary to a_w, the acidity of the product enhanced *Salmonella* inactivation without changing the overall shape inactivation behaviour of the pathogen towards a_w and storage temperature, indicating these were the main factors influencing the shape of the curve of *Salmonella* during storage. Interestingly, a linear relationship was observed when plotting the ratio of δ predicted by the global model from acid and low-

acid DFS with the same a_w versus storage temperature (Figure 2) and it was quantified through a linear relationship described by Eq. 3 with a goodness-of-fit of R^2_{adj} of 0.964.

$$\frac{\delta_{acid}}{\delta_{low-acid}} = 0.4494 + 0.0138 \cdot T \quad \text{Eq. 3}$$

where δ_{acid} and $\delta_{low\text{-}acid}$ are the δ values predicted by the global model for acid DFS (Table 2) and T is the storage temperature.

In acid DFS, the time for the first Log reduction of *Salmonella* decreased by 50, 41, 34 and 21% at storage temperatures of 4, 10, 15 and 25°C, respectively, in comparison with the values found in low-acid DFS, indicating that the effect of the acidification during the DFS production (leading to different levels and physiological status of the pathogen) on the subsequent *Salmonella* inactivation was stronger at lower storage temperatures.

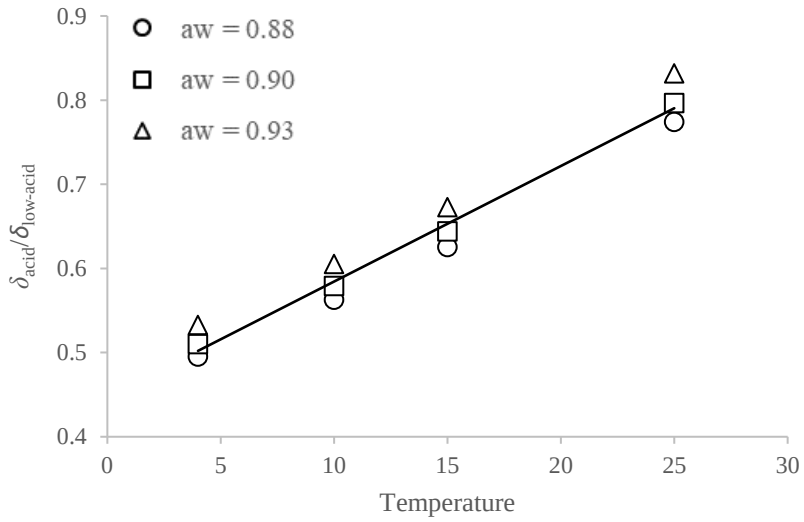

Figure 2. Ratios of predicted δ of low-acid and acid dry fermented sausages with different a_w and stored at different storage temperatures (4, 10, 15 and 25 ˚C). The line shows the fit of the linear model according to Eq. 3.

Regarding a_w and although the ratio δ_{acid} / $\delta_{low\text{-}acid}$ was systematically higher in sausages with higher a_w, it was not statistically different from ratio of δ found in sausages with lower a_w at the same storage temperature (p-value>0.05), indicating that the ratio of δ was not significantly affected by the a_w when sausages were stored at the same temperature. Thus, the effect of acidity on the first Log reduction of *Salmonella* in DFS was suggested to be mainly dependent on the storage temperature but not on the a_w.

3.6 Assessment of the predictive performance of the developed models

Only a few scientific studies are available regarding the behaviour of *Salmonella* during the storage of DFS. In these studies considerably different fermentation and drying conditions, diameter and sausage formulation were used, which are reported to affect the inactivation of *Salmonella* in DFS (Mataragas et al., 2015), hindering the comparison of the pathogen reduction loads reported by literature with the ones obtained in the present study. Moreover, in most of them, *Salmonella* was inoculated into ripened DFS, thus, without taking into account the effect of the progressive adaptation to the harsh product characteristics on the *Salmonella* behaviour during the storage period.
Low-acid and acid DFS with characteristics and physicochemical parameters similar to the ones assessed in the present study were studied by Gunvig et al. (2016) and the *Salmonella* counts obtained during the

storage of these products were used as totally independent data to evaluate the predictive performance of the developed models (Supplementary Tables 2 and 3). Results showed that for low-acid DFS, 65/115 (62%) of the predictions obtained with the developed model were within the ASZ (± 1 Log) (Supplementary Table 2). It is worth to highlight that most of the residuals obtained with the comparison of the observed and predicted *Salmonella* counts were negative, especially for temperatures above 16 °C, indicating that the model provided slightly fail safe predictions. On the other hand, this trend was not observed at 5°C, where slightly/or no inactivation of *Salmonella* was expected. These results could be explained by the conservative pH values (i.e. worst case scenario, pH 5.6-5.7) of the DFS used in the present study for developing the model, which were slightly higher than those of the DFS (pH 5.1-5.6) used for the evaluation of the predictive performance of the model for low-acid DFS. Regarding the prediction of *Salmonella* counts in acid DFS, 94/117 (80 %) of the predictions were within the ASZ (± 1 Log) (Supplementary Table 3), indicating a good predictive performance of the model developed for acid DFS.
Overall, results showed the good predictive performance of the models and reported evidences that models could be an objective and reliable tool to calculate the *Salmonella* reduction by the application of a corrective storage period.
The developed model quantified the inactivation of *Salmonella* during the storage of DFS with different physicochemical properties (i.e. different a_w and pH at the beginning of storage). The greatest strength of the model lies in the experimental design of the study, which through the simulation of a *Salmonella* contamination in the raw materials, takes into account the harsh conditions of the processing process.

3.7 Application of the developed models

The bactericidal effect against *Salmonella* observed during the storage of DFS could be used for sausage manufacturers as a lethality treatment to enhance the *Salmonella* inactivation in the product before being released into the market, particularly if suspected to be contaminated with the pathogen. The predictive models developed in this study would assist manufacturers to set the necessary time and temperature to achieve the desired reduction of *Salmonella* in different types of sausages (low-acid and acid) as a function of the a_w of the finished product. In this framework, the developed model predicts that a short corrective storage time of 5 to 8 d (depending on the a_w of the DFS) would let to a 1 Log reduction of the *Salmonella* concentration in acid DFS. Overall, and considering the estimated shelf-life of the fermented sausages, the application of a such corrective storage time immediately after the drying process and before the commercialization of the product could be used by sausage manufacturers as a control measure to enhance the reduction of *Salmonella* levels.

4 Conclusions

Dry fermented sausage manufacturers can take advantage of the time-temperature conditions of the storage and the physicochemical characteristics of the product, mainly a_w, to further enhance *Salmonella* inactivation. For this purpose, the developed models quantifying the bactericidal effect of the temperature and low a_w during the storage of DFS can be used by food manufacturers as a risk management tool to design a corrective storage and hence, to establish a risk minimization strategy to enhance *Salmonella* reduction when the fermentation and drying processes are not enough to reduce the levels of *Salmonella* in the product.

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7 Supplementary data

Available at: https://doi.org/10.1016/j.ijfoodmicro.2021.109160

DISCUSSION

5. Discussion

This section comprehensively discusses the results reported in the different Articles included in the PhD report, grouped in three blocks (Figure 8):

1. Behaviour of pathogens in RTE meat products without the application of any intervention strategy to control pathogenic bacteria (control, baseline scenario).
2. Behaviour of pathogens in RTE meat products in response to intervention strategies aiming to inhibit growth (antimicrobials and packaging), inactivate (HPP, corrective storage) and/or in response to combined intervention strategies to explore additive, synergistic or antagonistic interactions.
3. Contribution of the intervention strategies when applied as control measures on the compliance of performance and food safety objectives.

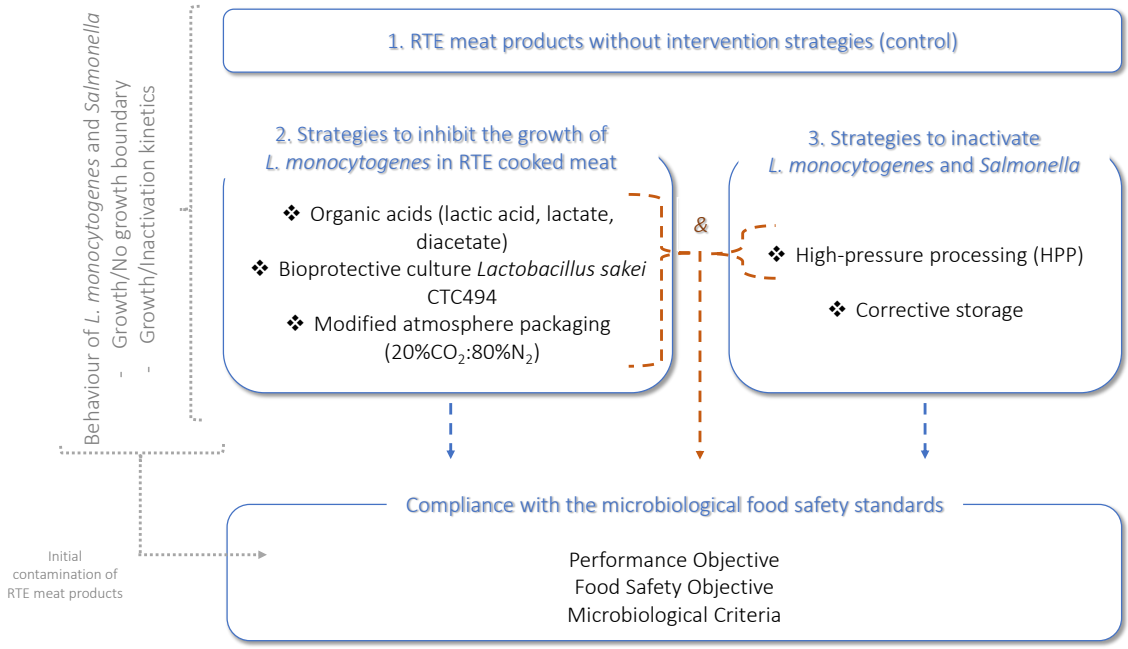


Figure 8. Flow diagram of the organization of the general discussion of the results.

5.1. Behaviour of pathogens in RTE meat products under control (baseline) conditions

5.1.1 *Listeria monocytogenes*

The predicted growth/no growth behaviour of *L. monocytogenes* during the storage was estimated through the gamma concept approach for different RTE meat products, including raw, cooked and dry-cured meat products. Figure 9 and Table 12 show the distribution of the pH and a_w of the products used in the studies of this PhD thesis and the associated growth probability of *L. monocytogenes* predicted as described in section 3.2.1. The figure also shows the combination of values set by the Regulation (CE) 2073/2005 (European Commission, 2005) to categorize RTE foods as able (a_w>0.92) and unable (a_w≤0.92) to support

the growth of *L. monocytogenes* and the predicted boundaries for 10, 50 and 90 % probability of growth at 3.9 and 9.5 °C.

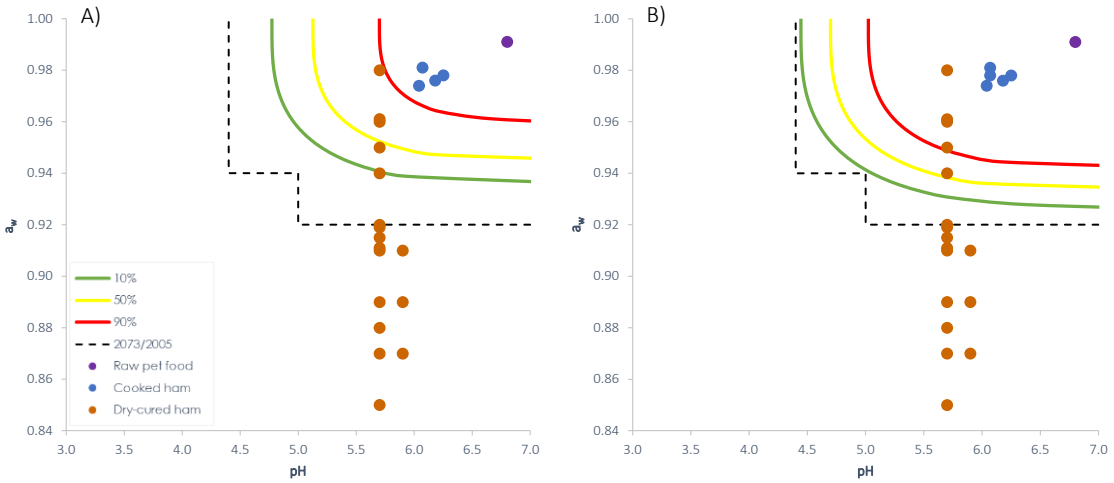


Figure 9. Growth probability of *L. monocytogenes* in control RTE meat products predicted by the gamma approach considering the pH and a_w of the products and a storage temperature of 3.9 °C (A) and 9.5 °C (B). Continuous lines correspond to the isoprobability lines at 10% (green), 50% (yellow) and 90% (red) predicted by the model of Augustin et al. (2005). Dashed lines correspond to threshold pH and a_w values set by the European Commission (2005) to categorize RTE foods as able (a_w>0.92) and unable (a_w≤0.92) to support the growth of *L. monocytogenes*.

In the case of **raw and cooked RTE meat products**, the gamma values for the pH and a_w were close to 1 (Table 12), indicating that pH and a_w values allowed 87-99% and 71-94%, respectively, of the optimal growth rate of *L. monocytogenes* (ICMSF, 1996). On the other hand, the gamma values for the storage temperatures were close to 0, allowing only 2% (at 3.9 °C) and 9% (at 9.5 °C) of the optimal growth rate of the pathogen, which indicates that storage temperature was the factor with a major impact on reducing *L. monocytogenes* growth under the conditions tested. Nevertheless, the predicted growth probability of *L. monocytogenes* in control RTE raw and cooked meat products was above the 90% boundary (Figure 9), ranging from 91 to 96% at 3.9 °C and up to 100% at 9.5 °C (Table 12). These predictions were in line with the observed ability of *L. monocytogenes* to grow in control raw and cooked meat products (vacuum-packed) from Articles 6, 8 and 9.

For conditions supporting the growth of *L. monocytogenes* the growth kinetics was determined. As shown in Figure 10, the storage temperature in the range of 2-20 °C highly affected the growth rate of *L. monocytogenes*. The secondary modelling performed in Articles 6 and 9 showed that the transformed squared root growth rate of the pathogen linearly decreased with lowering temperature (Figure 11). The slopes of the secondary models were practically the same, indicating a similar impact of temperature on the growth rate of *L. monocytogenes* in control cooked ham in both Articles 6 and 9 (Figure 11).

Besides the reasonably foreseeable conditions to which the product will be submitted to (regarding e.g., temperature), the inherent variability of the product (intrinsic factors) should be considered when determining the safe shelf-life of the product. In this sense, results showed that the secondary model developed in Article 6 was within the 95th confidence interval associated with that of Article 9 (Figure 11), indicating not statistically significant differences. On the other hand, the growth rates of *L. monocytogenes* in commercial control cooked ham from Article 8 (from which the exact formulation was not known) were of the same order of magnitude but slightly lower than the growth rates observed for the *ad-hoc* manufactured cooked ham, which may be explained by the effect of non-controlled factors. These results highlighted the need to determine the safe shelf-life of foods through a product oriented-approach.

Table 12. Growth probability of *L. monocytogenes* in control RTE meat products predicted by the gamma approach considering the pH and a_w of the products included in studies of this PhD and storage temperatures of 3.9 °C and 9.5°C.

Article	Type of product[a]	Physicochemical characteristics		γ			Growth probability (%)	
		pH	a_w	pH	a_w	Temperature 3.9 / 9.5 °C	At 3.9 °C	At 9.5 °C
1	RPFD	6.80	0.991	0.99	0.94	0.02 / 0.09	**95.8**	**99.8**
9	CH	6.07	0.978	0.87	0.76	0.02 / 0.09	**92.5**	**99.6**
6	CH	6.07	0.981	0.87	0.80	0.02 / 0.09	**92.9**	**99.6**
7&8	CH	6.25	0.978	0.92	0.76	0.02 / 0.09	**93.8**	**99.7**
7&8	CH	6.18	0.976	0.90	0.74	0.02 / 0.09	**93.0**	**99.6**
4	CH	6.04	0.974	0.87	0.71	0.02 / 0.09	**91.2**	**99.5**
10	DCH	5.70	0.980*	0.76	0.79	0.02 / 0.09	**86.3**	**99.2**
10	DCH	5.70	0.961*	0.76	0.53	0.02 / 0.09	**69.2**	**97.8**
10	DCH	5.70	0.960*	0.76	0.51	0.02 / 0.09	**67.0**	**97.6**
10	DCH	5.70	0.950*	0.76	0.37	0.02 / 0.09	**35.4**	**91.6**
10	DCH	5.70	0.940*	0.76	0.24	0.02 / 0.09	**6.9**	**59.5**
10	DCH	5.70	0.920*	0.76	0.00	0.02 / 0.09	0.0	0.0
10	DCH	5.70	0.919*	0.76	0.00	0.02 / 0.09	0.0	0.0
10	DCH	5.70	0.915*	0.76	0.00	0.02 / 0.09	0.0	0.0
10	DCH	5.70	0.911*	0.76	0.00	0.02 / 0.09	0.0	0.0
10	DCH	5.70	0.890*	0.76	0.00	0.02 / 0.09	0.0	0.0
10	DCH	5.70	0.870*	0.76	0.00	0.02 / 0.09	0.0	0.0
11	DCH (Ib)	5.70	0.910	0.76	0.00	0.02 / 0.09	0.0	0.00
11	DCH (Ib)	5.70	0.880	0.76	0.00	0.02 / 0.09	0.0	0.0
11	DCH (Ib)	5.70	0.850	0.76	0.00	0.02 / 0.09	0.0	0.0
11	DCH (Se)	5.90	0.910	0.83	0.00	0.02 / 0.09	0.0	0.0
11	DCH (Se)	5.90	0.890	0.83	0.00	0.02 / 0.09	0.0	0.0
11	DCH (Se)	5.90	0.870	0.83	0.00	0.02 / 0.09	0.0	0.0

[a]RPFD: Raw pet food for dog; CH: Cooked ham; DCH: Dry-cured ham (Ib: Iberian; Se: Serrano)
*values of a_w adjusted to the CCD requirement

Another important aspect to consider when determining the safe shelf-life of foods, is the strain and biological variability of the pathogen. According to Arygi et al. (2015), the effect of the *L. monocytogenes* strain variability could be high, explaining up to 1/3 of the variability in the *L. monocytogenes* growth found in literature. Thereby, with the aim to cover worst-case (conservative) scenarios, i.e., scenarios favourable for *L. monocytogenes* growth, the safe shelf-life of foods should be determined through the study of the behaviour of a *L. monocytogenes* strain with a high growth ability. In this respect, results from Article 6 showed that the strain CTC1034 grew faster than the cocktail consisting of strains recommended by the EU Reference Laboratory for *L. monocytogenes* (12MOB045Lm and 12MOB089Lm) (EURL-Lm, 2021) and the clinical isolate Scott A. Moreover, the growth rate of *L. monocytogenes* CTC1034 strain was higher than that predicted by the predictive model (Mejlholm & Dalgaard, 2009) available in the Food Spoilage and Safety Predictor (FSSP) software (http://fssp.food.dtu.dk) (Article 6). Therefore, the results of this PhD thesis provide scientific data to support the suitability of *L. monocytogenes* strain CTC1034 in validation studies for determining the safe shelf-life of RTE cooked meat products.

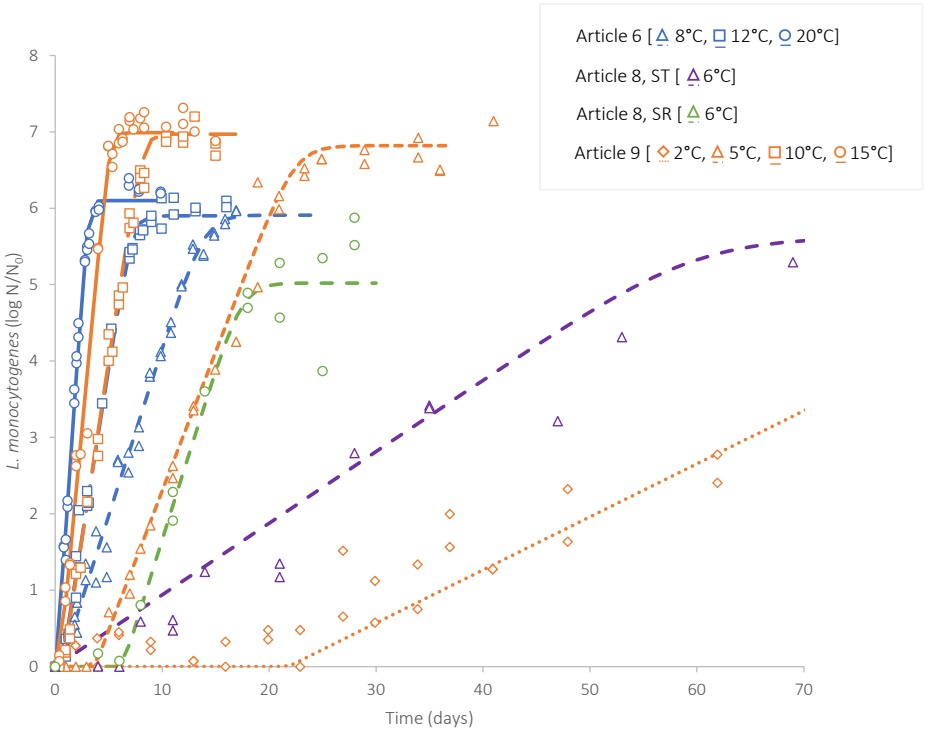


Figure 10. Growth of *L. monocytogenes* in control RTE cooked meat products, including vacuum-packed commercial (Article 8) and *ad-hoc* manufactured at IRTA (Article 6 and 9) stored at 2-20 °C. Dots correspond to the observed log increase and lines to the fit of the Log-logistic primary growth model used to estimate the growth rate. ST: Standard formulation; SR: Sodium Reduced.

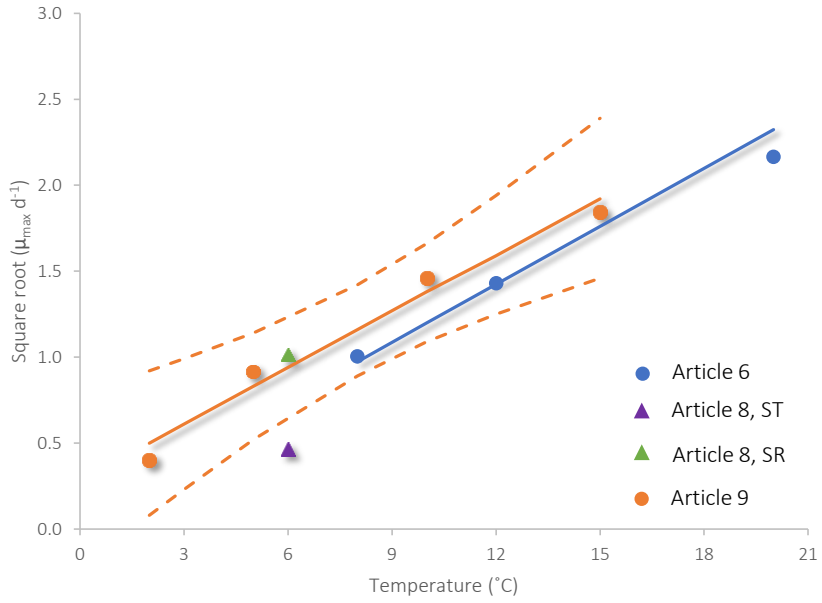


Figure 11. Impact of temperature on the growth rate (μ_{max}) of *L. monocytogenes* in control vacuum-packed cooked ham. Dots correspond to the square root of observed μ_{max} in commercial (triangles) and in *ad-hoc* manufactured at IRTA (circles) cooked ham. Continuous lines correspond to the secondary model fit. In dashed lines, the confidence interval at 95% of the secondary model obtained in Article 9. ST: Standard formulation; SR: Sodium reduced formulation.

In the case of **RTE dry-cured meat products**, the results obtained with the gamma approach showed that a_w was the main factor enabling or restricting the growth of *L. monocytogenes*, with predicted growth of the pathogen in dry-cured meat with a_w of 0.94 (γ >0) and no growth for a_w of 0.92 (γ =0) (Figure 9, Table 12). The no-growth predictions agreed with the findings of Article 11, where no growth of *L. monocytogenes* was observed in Iberian dry-cured hams (pH of 5.7) with a_w values of 0.85, 0.88 or 0.91 nor in Serrano dry-cured hams (pH of 5.9) with a_w values of 0.87, 0.89 or 0.91 stored at 2, 8, 15 or 25 °C. Under these non-growing conditions *L. monocytogenes* tended to die. The higher the storage temperature the higher the inactivation of *L. monocytogenes* along the storage of dry-cured ham (Article 11). In the same line, Cava et al. (2020; 2021) and Porto-Fett et al. (2008) reported that the inactivation of *L. monocytogenes* in RTE dry-cured/fermented meat products (dry-cured loin, dry-fermented sausages "salchichón", "chorizo" and soudjouk-style) was faster at a high storage temperature (e.g., room temperature) compared to low temperature (e.g., refrigeration temperature). According to Leistner & Gorris (1995), this could be explained by the metabolic exhaustion phenomenon, which occurs in the situation where vegetative bacteria cannot grow and their inactivation is enhanced when the conditions are close to the growth/no growth limit for the microorganism, such as in the storage of the product at room temperature. On the other hand, results showed that *L. monocytogenes* was able to survive (without significant inactivation) in dry-cured ham with a_w of 0.91 stored at 2 °C for 90 days (Article 11). The occurrence and ability of *L. monocytogenes* to survive in RTE dry-cured meat products may compromise the compliance of the microbiological criteria established for *L. monocytogenes* in these types of products, especially those that are regulated under a zero-tolerance policy (Section 1.2).

Overall, these results highlighted the impact of a_w on restricting the growth of *L. monocytogenes* and the role of storage temperature on enhancing the inactivation of the pathogen in RTE dry-cured meat products under non-growing conditions. In this respect, food business operators of RTE dry-cured meat products can consider the identified growth/no growth a_w boundary value to set a critical limit within the HACCP plan with the aim of proving that dry-cured meat products can be classified as RTE foods unable to support the growth of *L. monocytogenes*.

5.1.2 *Salmonella*

Figure 12 and Table 13 show the distribution of the pH and a_w of the meat products used in the studies of this PhD thesis and the associated growth probability of *Salmonella* predicted as described in section 3.2.1. The figure also shows the predicted boundaries for 10, 50 and 90% probability of growth at 9.5 °C.
Temperature and a_w were the main factors restricting the growth of *Salmonella* in RTE meat products at 9.5 °C, with gamma values closer to 0 compared to gamma values of pH (Figure 12). RTE meat products with $a_w \geq 0.96$ would support the growth of *Salmonella* with a growth probability increasing from 9% (a_w of 0.96) to 97% (a_w of 0.99) (Figure 12, Table 13). Under these conditions, the main factor limiting the growth of *Salmonella* was temperature (γ_T=0.3) indicating that at 9.5 °C the growth would be 30% of the maximum growth rate of the pathogen at optimum temperature (Table 13).

On the other hand, for **RTE dry-cured meat products** with $a_w < 0.96$, no growth was predicted, due to γ_{a_w} = 0. This was in agreement with Article 12 dealing with dry-fermented sausages stored at 4, 10, 15 or 25 °C. Under these non-growing conditions, *Salmonella* was more inactivated with increasing the storage temperature, which may be related with the metabolic exhaustion phenomenon (Leistner & Gorris, 1995), as explained for *L. monocytogenes*. In fact, under refrigeration conditions, *Salmonella* could survive without losing viability in low-acid dry-fermented sausages with a_w of 0.88 stored at 10 °C for at least 30 days (Article 12).

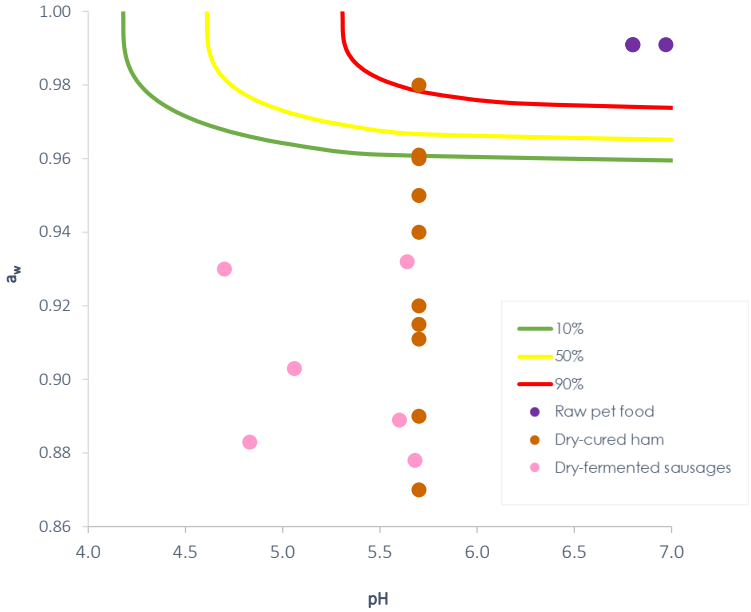


Figure 12. Growth probability of *Salmonella* in control RTE meat products predicted by the gamma approach considering the pH and a_w of the products and a storage temperature of 9.5 °C. Continuous lines correspond to the isoprobability lines at 10% (green), 50% (yellow) and 90% (red) predicted by the model of Augustin et al. (2005).

Table 13. Growth probability of *Salmonella* in control RTE meat products predicted by the gamma approach considering the pH and a_w of the products included in studies of this PhD and storage temperature of 9.5 °C.

Article	Type of product[a]	Physicochemical characteristics		γ			Growth probability (%)
		pH	a_w	pH	a_w	Temperature 9.5 °C	At 9.5 °C
1 &3	RPFD RPFC	6.80	0.991	1.00	0.91	0.03	**96.7**
2	RPFD	6.97	0.991	1.00	0.91	0.03	**96.7**
10	DCH	5.70	0.980*	0.86	0.67	0.03	**91.3**
10	DCH	5.70	0.961*	0.86	0.23	0.03	**12.6**
10	DCH	5.70	0.960*	0.86	0.21	0.03	**8.7**
10	DCH	5.70	0.950*	0.86	0.00	0.03	0.0
10	DCH	5.70	0.940*	0.86	0.00	0.03	0.0
10	DCH	5.70	0.920*	0.86	0.00	0.03	0.0
10	DCH	5.70	0.919*	0.86	0.00	0.03	0.0
10	DCH	5.70	0.915*	0.86	0.00	0.03	0.0
10	DCH	5.70	0.911*	0.86	0.00	0.03	0.0
10	DCH	5.70	0.890*	0.86	0.00	0.03	0.0
10	DCH	5.70	0.870*	0.86	0.00	0.03	0.0
12	DFS	5.64	0.932	0.84	0.00	0.03	0.0
12	DFS	4.70	0.930	0.54	0.00	0.03	0.0
12	DFS	5.06	0.903	0.68	0.00	0.03	0.0
12	DFS	5.60	0.889	0.83	0.00	0.03	0.0
12	DFS	4.83	0.883	0.60	0.00	0.03	0.0
12	DFS	5.68	0.878	0.85	0.00	0.03	0.0

[a]RPFD: raw pet food for dog; RPFC: raw pet food for cat; DCH: Dry-cured ham; DFS: Dry-fermented sausages

*values of a_w adjusted to the CCD requirement

In case of raw pet food, though the predicted growth probability was very high at 9.5 °C, in practice, the frozen (-18 °C) and refrigerated (4 °C during thawing) storage would actually inhibit the growth of the pathogen (Articles 1, 2, 3).

Overall, results showed that the survival capability of *Salmonella* in RTE meat products observed in the studies carried in products with low a_w, and especially under refrigeration, compromises the compliance of the microbiological criteria (no detection of *Salmonella*) and also pose a risk to consumer's health due to the low infective dose of the pathogen (Section 1.3). Therefore, strategies aiming to eliminate or reduce the levels of *Salmonella* would be needed to enhance the microbiological safety of RTE meat products.

5.2. Antimicrobial strategies aiming to inhibit the growth of pathogens in RTE cooked meat products

The impact of three different antimicrobial strategies aiming to inhibit the growth of *L. monocytogenes* in cooked meat products was evaluated and compared (Table 14) including (i) the use of organic acid salts, e.g., lactate and/or diacetate (Article 6), (ii) the addition of the bioprotective culture *L. sakei* CTC494 (Article 9) and (iii) the use of MAP (Article 8). For (i) and (ii), vacuum packaging was applied.

Results from challenge tests dealing with the behaviour of *L. monocytogenes* in cooked hams formulated with a concentration of salts of **organic acids** with minimal or acceptable effects on the sensory characteristics of the product and close to the growth/no growth boundary of the pathogen showed that *L. monocytogenes* was not able to grow in cooked ham formulated with the combination of 2% lactate and 0.45% diacetate stored at 12 and 20 °C or with the combination of 4% lactate or 2% lactate and 0.11% diacetate stored at temperatures ≤12 °C (Article 6, Table 14). Therefore, the results obtained in Article 6 proved that the strategy designed through predictive microbiology approach was useful and challenge testing helped to validate the behaviour of *L. monocytogenes* in worst-case scenarios. This methodology gives value to predictive microbiology and challenge testing as useful and complementary approaches to help food business operators to determine the concentration of organic acids necessary to obtain an RTE product classified in the category 1.3 of the microbiological criteria Regulation (CE) 2073/2003 (European Commission, 2005) as not able to support the growth of *L. monocytogenes*, for which the safe shelf-life regarding this pathogen is not a concern, i.e., safety by design.

Listeria monocytogenes was able to grow in cooked hams formulated with lower concentrations of organic acids and/or at high storage temperatures, though in most of the cases with an extended lag time and a reduced growth rate compared to control (cooked hams formulated without organic acids) which lead to an impact on the safe shelf-life ranging from 3.5 to 9-fold or a total inhibition of the growth of the pathogen (i.e. making it not relevant for safe shelf-life) (Table 14).

The application of the **bioprotective culture** *L. sakei* CTC494 (Article 9) did not affect the lag time and growth rate of the pathogen but had an impact on the maximum population density (N_{max}) reached by *L. monocytogenes,* which is a key parameter determining the risk of listeriosis (Pérez-Rodríguez et al., 2017). The N_{max} was from 2 to 7.5 log units lower with the application of *L. monocytogenes*: *L. sakei* CTC494 ratios of 1:3 and 1:5 compared with that of the pathogen in cooked ham without the bioprotective strain (monoculture conditions). The magnitude of the pathogen inhibition increased by increasing the amount of bioprotective strain. In this respect, the application ratio of at least 1:3 (i.e., initial log concentration of the bioprotective strain 3-fold higher than the initial log concentration of the pathogen) was required to ensure the efficacy of the *L. sakei* CTC494 at 15 °C.

Table 14. Qualitative and quantitative summary of the impact of the intervention strategies on *L. monocytogenes* behaviour in cooked ham.

Article	Cooked ham (CH)[a]	Intervention strategy		T (°C)	Behaviour of *L. monocytogenes* compared with the control[b]	Impact on the safe shelf-life[d] in relation to the control product
		Type	Amount			
6	CH1	Organic acids	2.8% lactate	20	Lag time extended by 2 days; growth rate reduced by 68%	From 2 to 7 days (x3.5)
			4 % lactate	20	Lag time extended by 5 days; growth rate reduced by 88%	From 2 to 18 days (x9)
			2% lactate + 0.11% diacetate	20	Lag time extended by 2 days; growth rate reduced by 85%	From 2 to 13 days (x6.5)
			2% lactate + 0.45% diacetate	20	Growth totally inhibited. Inactivation	From 2 days to not constraining[e]
			4 % lactate	12	Growth totally inhibited. Inactivation	From 4 days to not constraining[e]
			2% lactate + 0.45% diacetate	12	Growth totally inhibited. Inactivation	From 4 days to not constraining[e]
			2.8% lactate	8	Lag time extended by 43 to 49 days; growth rate reduced by 54 to 71%	From 8 to 61 days (x7.6)
			2% lactate + 0.11% diacetate	8	Growth totally inhibited. Inactivation	From 8 days to not constraining[e]
9	CH2	*L. sakei* CTC494	Ratio 1:1[c]	5-15	Slightly or not significant reduction of maximum population density	No impact
				2	Reduction of maximum population density by 2.8 log	No impact
			Ratio 1:3[c]	5-15	Reduction of maximum population density by 1.9 to 3.7 log	No impact
				2	Growth totally inhibited. Inactivation	From 71 days to not constraining[e]
			Ratio 1:5[c]	5-15	Reduction of maximum population density by 4.0 to 5.9 log	No impact
				2	Growth totally inhibited. Inactivation	From 71 days to not constraining[e]
8	CH3 (A, ST)	MAP	20% CO_2; 80% N_2	6	Lag time extended by 10 days; growth rate reduced by 19%	From 25 to 38 days (x1.5)
	CH4 (A, SR)	MAP	20% CO_2; 80% N_2	6	Lag time extended by 7 days; growth rate reduced by 21%	From 18 to 28 days (x1.5)
	CH5 (B, ST)	MAP	20% CO_2; 80% N_2	6	Lag time extended by minimum 5 days; no impact on growth rate	From 36 to 40 days (x1.1)
	CH6 (B, SR)	MAP	20% CO_2; 80% N_2	6	No impact on lag time; growth rate reduced by 60%	From 14 to 24 days (x1.7)

[a] Products used in Articles 6 (CH1) and 9 (CH2) were manufactured *ad-hoc* at IRTA pilot plant (standard formulation). Cooked hams used in Article 8 were commercial products obtained directly from two different producers (A and B) and included products with a standard formulation (ST) and with a sodium-reduced (SR) formulation.

[b] *L. monocytogenes* CTC1034 was used in all the studies. In addition, a cocktail of *L. monocytogenes* strains (12MOB045LM, Scott A and 12MOB089LM) was used in Article 6. Control conditions were: for Article 6 vacuum-packed cooked ham without added organic acid salts; for Article 9 *L. monocytogenes* in monoculture (i.e. without bioprotective strain) in vacuum-packed cooked ham; for Article 8: vacuum-packed cooked ham.

[c] Ratio *L. monocytogenes: L. sakei* CTC494.

[d] Safe shelf-life of products was estimated as the time needed for *L. monocytogenes* to reach 100 cfu/g considering an initial level of the pathogen of -1.4 cfu/g. In brackets the extension of safe shelf-life compared to the control product thanks to the application of the antimicrobial strategy.

[e] Shelf-life not dependent on the growth of *L. monocytogenes.*

From the industrial point of view, the application of this ratio is reasonable and easily achievable considering that the initial contamination of the product by *L. monocytogenes* is generally lower than 10 cfu/g (EFSA BIOHAZ Panel, 2018). The storage temperature was the other relevant factor influencing the inhibitory effect. The lower the temperature the stronger the bioprotective effect of *L. sakei* CTC494 and at 2 °C a total inhibition of *L. monocytogenes* growth (with a slight inactivation) was observed from the very beginning, even before the bioprotective culture reached the stationary phase. The inhibitory effect could

be satisfactorily characterized through the mathematical model based on the Jameson-effect, with a temperature-dependent interaction factor for storage temperatures < 5 °C, describing the growth/inactivation of *L. monocytogenes* after *L. sakei* reached its maximum population density.
The mathematical model presented in Article 9 satisfactorily predicted the fate of both the bioprotective strain CTC494 and *L. monocytogenes* in vacuum-packed cooked ham under dynamic conditions of temperature (non-isothermal), including abusive temperature, thus proving to food business operators and stakeholders its suitability to assess and optimize the conditions of application of this bioprotective strategy.

The third strategy assessed to limit the growth of *L. monocytogenes* in cooked meat products was the use of **modified atmosphere packaging** (MAP) with 20% CO_2 as antimicrobial gas and without O_2 (i.e., 80% N_2) (Article 8). For most of the commercial cooked hams, the lag time of *L. monocytogenes* was extended and the growth rate was reduced under MAP compared to vacuum conditions, which lead to an impact on the safe shelf-life ranging from 1.1 to 1.7-fold (Table 14). Despite the impact of MAP on the safe shelf-life was lower than that observed for organic acids, it could be a useful intervention strategy to use to *control L. monocytogenes* in cooked ham that would be in line with the increasing demand by consumer of products containing less additives.

5.3. Post-lethality strategies to reduce the levels of pathogens in RTE meat products

5.3.1 HPP for RTE meat products

The meta-analysis of the entire set of inactivation data of *L. monocytogenes* and *Salmonella* by HPP reported in Articles 1-8 and 10 indicated that the HPP technological parameters (pressurization level and holding time), the product a_w, the type and amount of antimicrobial added in the product and the enumeration time after HPP were the statistically significant factors determining the extent of the HPP inactivation of pathogens in RTE meat products. On the other hand, the initial temperature of the pressurization fluid, the pathogen (species), the physiological state of the strain before HPP, the type of product and the packaging system were not statistically significant (Table 15).
In the following sections the significance of the evaluated factors on the efficacy of HPP is discussed.

5.3.1.1. Impact of HPP technological parameters

As extensively reported in the literature (EFSA BIOHAZ Panel, 2022), a higher inactivation of both *L. monocytogenes* and *Salmonella* was observed with increasing pressure time and holding time of the HPP treatment, both factors resulting statistically significant when the entire set of data of all the studies was meta-analysed (Table 15). However, for some products and HPP conditions, the pathogen after HPP was not detected (i.e., levels below the detection limit) and thus it was uncertain (not quantifiable) whether a higher pressure or longer time would result in a higher inactivation of the pathogen. On the other hand, the HPP kinetics showed non-linear trends in some cases. For instance, shoulders of no relevant inactivation during the first seconds/minutes of HPP were observed in *L. monocytogenes* strain CTC1011 in cooked ham pressurized at 400 MPa (Article 4) or in *L. monocytogenes* strain CTC1034 in some commercial cooked hams (Article 7), while tails of resistant cells without further inactivation with increasing holding time occurred in *Salmonella* strains CTC1022, GN0082 and GN0085 pressurized at 600 MPa (Article 1). The occurrence of tails was dependent on the strain, HPP treatment and composition of RTE meat product (Articles 1-4, 7). The tails have been related to the presence of two different subpopulations, the major one more susceptible to HPP, followed by the second subpopulation more resistant to HPP, responsible for the tail (Geeraerd et al., 2000).

Regarding the impact of the initial temperature of the pressurization fluid, pathogen inactivation has been reported to increase when temperatures are above ambient temperature (Hogan et al., 2005). All studies carried out in this PhD used an initial temperature of the pressurization fluid far below to 25 °C (as usually applied industrially), which would not result in thermal effects and therefore, it could be the reason why this factor was not statistically significant according to the meta-analysis model (Table 15).

Table 15. Statistical significance (*p*-values)[a] obtained for each fixed factor included in the meta-analysis models of the inactivation data of *L. monocytogenes* and *Salmonella* by HPP.

Fixed factors	Model 1[b] [All data] n=1332	Model 2 [All data] n=1332	Model 3 [$a_w \geq 0.95$] n=1256	Model 4 [$a_w \geq 0.96$] n=1240	Model 5[c] [$a_w < 0.96$] n=92
Pressure (MPa)	$<2.2 \cdot 10^{-6}$	$<2.2 \cdot 10^{-6}$	$<2.2 \cdot 10^{-6}$	$<2.2 \cdot 10^{-6}$	$<2.2 \cdot 10^{-6}$
Time (min)	$<2.2 \cdot 10^{-6}$	$<2.2 \cdot 10^{-6}$	$<2.2 \cdot 10^{-6}$	$<2.2 \cdot 10^{-6}$	NA[e]
Temperature (°C)	$3.02 \cdot 10^{-1}$	$1.09 \cdot 10^{-1}$	$9.43 \cdot 10^{-2}$	$1.12 \cdot 10^{-1}$	NA[e]
Pathogen	$6.47 \cdot 10^{-1}$	$6.36 \cdot 10^{-1}$	$4.58 \cdot 10^{-1}$	$3.53 \cdot 10^{-1}$	$5.74 \cdot 10^{-1}$
Physiological state	$6.06 \cdot 10^{-1}$	$6.84 \cdot 10^{-1}$	$5.37 \cdot 10^{-1}$	$7.42 \cdot 10^{-1}$	NA[e]
Type of product	$8.55 \cdot 10^{-1}$	$7.22 \cdot 10^{-1}$	$6.18 \cdot 10^{-1}$	$8.68 \cdot 10^{-1}$	NA[e]
a_w	$<2.2 \cdot 10^{-6}$	$<2.2 \cdot 10^{-7}$	$1.35 \cdot 10^{-2}$	$7.70 \cdot 10^{-1}$	$1.1 \cdot 10^{-9}$
pH	$4.20 \cdot 10^{-5}$	0.9643	$9.03 \cdot 10^{-1}$	$9.59 \cdot 10^{-1}$	NA[e]
Type of antimicrobial	$4.30 \cdot 10^{-7}$	NC[d]	NC[d]	NC[d]	NC[d]
Type and amount of antimicrobial	NC[d]	$1.3 \cdot 10^{-12}$	$1.1 \cdot 10^{-12}$	$2.80 \cdot 10^{-13}$	NA[e]
Formulation	$2.12 \cdot 10^{-1}$	$2.80 \cdot 10^{-1}$	$3.06 \cdot 10^{-1}$	$5.21 \cdot 10^{-1}$	NA[e]
Packaging system	$2.88 \cdot 10^{-1}$	$2.78 \cdot 10^{-1}$	$2.73 \cdot 10^{-1}$	$2.68 \cdot 10^{-1}$	NA[e]
Enumeration time	$9.20 \cdot 10^{-7}$	$6.30 \cdot 10^{-7}$	$2.07 \cdot 10^{-7}$	$1.20 \cdot 10^{-7}$	NA[e]

[a]Factors were considered significant when the *p*-value was below 0.05.
[b]In brackets, the subset of data. n: the number of log reduction data included in the model.
[c]The data used in this model belong to Article 10, covering the study of HPP on one single strain for each microorganism and in one type of RTE meat product. For this, a linear model with fixed effects was used instead of a mixed model with fixed and random effects (see section 3.2.2).
[d]NC: not considered in the model
[e]NA: not applicable, e.g., the factor had only 1 level.

5.3.1.2. Impact of pathogen and related factors

The distribution of the log reductions for the two **species** of pathogens studied, *L. monocytogenes* (n=35) and *Salmonella* (n=20) in raw and cooked RTE meat products formulated without antimicrobials and pressurized at 400 MPa for 5 min are shown in (Figure 13). For *L. monocytogenes*, log reduction data corresponds to the observed inactivation of 10 strains (12MOB045LM, 12MOB089LM, CTC1011, Scott A, CECT4031T, CTC1034, 12MOB102LM, CTC1769, 12MOB049LM and 12MOB050LM) in raw pet food (Article 1), 3 strains (CTC1011, CTC1034 and Scott A) in cooked ham (Article 4) and the predicted inactivation of CTC1034 strain in cooked ham under different packaging systems (Article 7). For *Salmonella*, log reduction data corresponds to the observed inactivation of 10 different strains (CECT702, CECT4565, CECT705, CTC1003, CTC1022, CECT34136T, CCUG21272, GN0085, GN0082 and CTC1756) in raw pet food (Article 1).

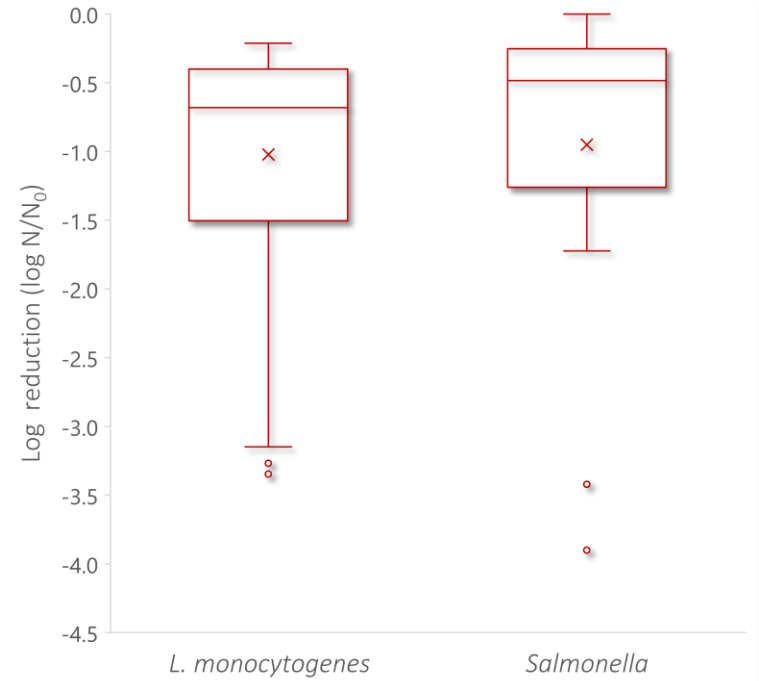


Figure 13. Distribution of HPP (400 MPa for 5min) inactivation (log reduction) data of *L. monocytogenes* (n=35) and *Salmonella* (n=20) in raw and cooked RTE meat products without antimicrobials (log reduction values extracted from Articles 1, 4 and 7).

In accordance with the lack of statistical significance of the factor "pathogen" in the meta-analysis model the mean log reduction between *L. monocytogenes* and *Salmonella* strains was very similar. However, the extent of inactivation was quite variable even when the same treatment intensity was applied (Figure 13), which can be attributed to the wide range of assessed conditions, i.e., different food matrixes, formulations and packaging systems.

Generally, Gram-positive bacteria such as *L. monocytogenes* have been described to be more piezo-resistant compared to Gram-negative bacteria such as *Salmonella* (Arroyo et al., 1997; Moreirinha et al., 2016). However, some studies have reported that Gram-negative bacteria were more piezo-resistant than Gram-negative bacteria in raw poultry meats, indicating that the ability of the microorganisms to withstand stresses is more related to strain-specific characteristics rather than the piezo-resistance given by the cell envelop of Gram-positive bacteria (den Besten et al., 2018).

In addition, the **strain** variability on the HPP resistance is also relevant regarding the shape of the inactivation curve, which can change the ranking of pressure resistance among strains when different HPP holding times are compared. In this respect, the kinetic study performed in cooked ham (Article 4) showed that *L. monocytogenes* strains CTC1011, CTC1034 and Scott A had different HPP-inactivation curves at 400 MPa (Figure 14). While CTC1011 had a convex HPP-inactivation curve with a shoulder of survival cells during the first minutes of treatment (being the most resistant strain for short HPP treatments), CTC1034 had a linear HPP-inactivation curve with constant inactivation along holding time and Scott A had a concave HPP-inactivation curve, indicating the occurrence of a tail of resistant cells at extended holding times, thus being the most resistant strain for long HPP treatments.
On the other hand, the study of inactivation kinetics of three *Salmonella* strains in non-acidulated raw pet food pressurized at 600 MPa showed that GN0085 had a slower inactivation rate compared to CTC1022 and GN0082 *Salmonella* strains (Article 1).

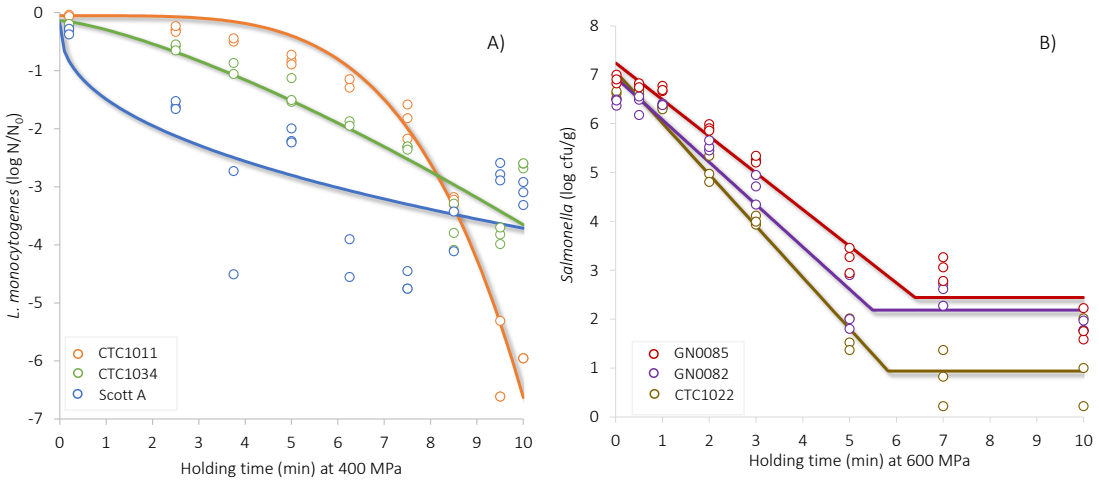


Figure 14. HPP inactivation kinetics of (A) *L. monocytogenes* strains in vacuum-packed cooked ham without organic acids and of (B) *Salmonella* in non-acidulated raw pet food for dog.

For the assessment and validation of the efficacy of HPP as control measure of the HACCP plan selected *L. monocytogenes* and *Salmonella* strains should be accurately selected to cover the worst-case scenario (EFSA BIOHAZ Panel, 2022). In this respect, this PhD thesis, a versatile cocktail of *L. monocytogenes* strains (CTC1011, CTC1034 and Scott A) with different HPP-inactivation curves (convex, lineal, and concave) and the *Salmonella* strain GN0085 with slow HPP inactivation rate, are proposed for challenge testing aiming to prove the accomplishment of the performance criteria for post-lethality treatments.

The **physiological state** associated with the growth phase and/or pre-culture conditions of the microorganisms prior HPP treatment is a key factor determining the resistance of microorganisms to HPP (EFSA BIOHAZ Panel, 2022). Assays dealing with raw and cooked RTE meat products were performed with *L. monocytogenes* and *Salmonella* cultures grown at 37 °C (until stationary phase) and subsequently frozen at -80 °C (Table 9). When used, they were thawed immediately before being inoculated to the product. On the other hand, in the assays dealing with dry-cured ham, bacterial cultures were grown at 37 °C and directly used without freezing (Table 9).

According to the output from the meta-analysis model, the physiological state of the strains determined by freezing at -80 °C was not a significant factor affecting the inactivation of microorganisms by HPP (Table 15). These results were in line with results reported by Serra-Castelló et al. (2023) (Figure 15), where the piezo-resistance of *L. monocytogenes* cultures (at stationary phase) grown at 37 °C without and with subsequent freezing at -80 °C was similar in hams with a_w of 0.88-0.98 and regardless of the pressure level applied. On the contrary, significant higher inactivation in ham with a_w of 0.98 by HPP at 400 and 600 MPa was observed when the pathogen was grown at cold temperatures to reach the stationary phase (adapted at 8 °C for 90 h) compared to those grown at 37 °C without or with subsequently freezing at -80 °C (Figure 15). These results are in line with those reported by Hereu et al. (2014) where the piezo-resistance of *L. monocytogenes* cultures grown at 37 °C with subsequently freezing at -80 °C in cooked ham and mortadella was higher compared to *L. monocytogenes* cultures grown at 8 °C prior HPP. In agreement with this, other studies also reported that *L. monocytogenes* was more resistant to HPP when the cultures were grown near the optimum growth temperature (37 °C) compared to cultures grown at cold temperatures (Bull et al., 2005; Hayman et al., 2007; Juck et al., 2012; McClements et al., 2001).

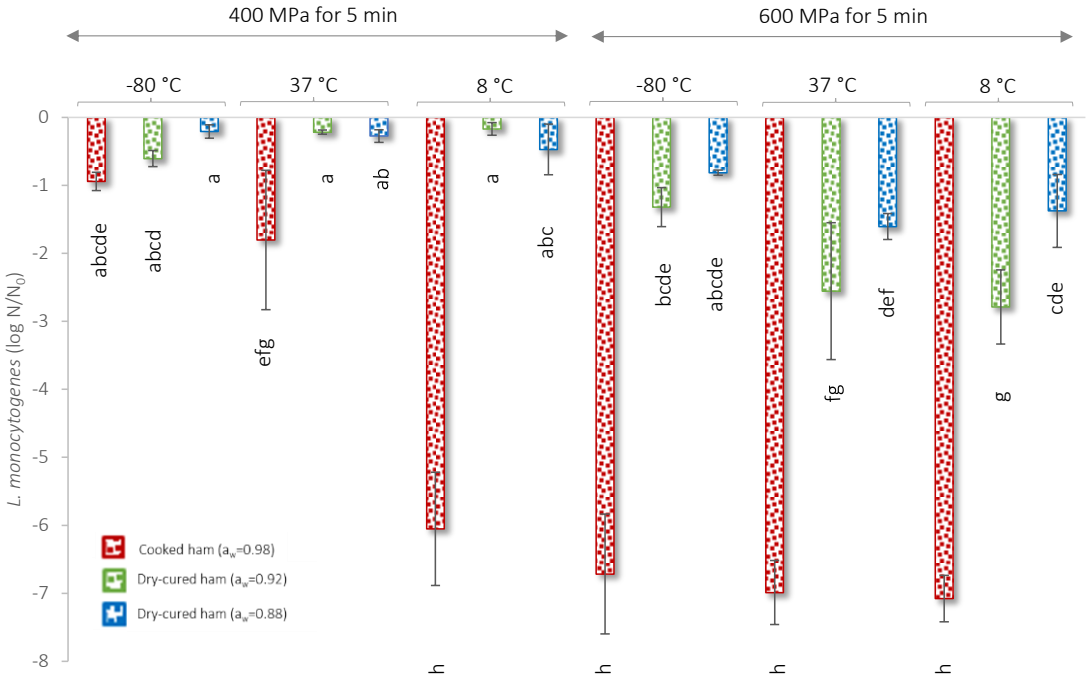


Figure 15. Inactivation of *L. monocytogenes* CTC1034 in RTE meat products pressurized at 400 MPa or 600 MPa for 5 min using inoculum with different physiological states grown till the early stationary phase at 37 °C for 18h and subsequently frozen at -80 °C; not frozen and growth at 8 °C for 90h (Serra-Castelló et al., 2023). Letters indicate significant differences (p-value <0.05) in pairwise comparisons from Tukey's test.

In practice, at industrial level, it is not possible to know the physiological state of the pathogens when RTE meat products are contaminated. Therefore, the assessment of the efficacy of intervention strategies through challenge testing is recommended to be conducted with inoculum with physiological states that confer resistance (ISO 20976-2, 2022). In this respect, *L. monocytogenes* cultures grown at 37 °C without or with subsequently freezing at -80 °C were the most appropriate for challenge testing in HPP validation studies for its higher piezo-resistance compared to *L. monocytogenes* cultures grown at cold temperatures. It is worth to highlight that this is opposite to the use of cold-adapted *L. monocytogenes* strains recommended by EURL-Lm (2021) for conducting challenge tests to assess the growth and shelf-life of products.

5.3.1.3. Impact of product characteristics

The meta-analysis models of log reduction data showed that the factors "type of product" (raw, cooked or dry) and "formulation" (standard or sodium-reduced) were not significant factors affecting the efficacy of HPP, but the "type of antimicrobial", "pH" and "a_w" were statistically significant (Table 15). However, when the "type and amount of antimicrobial" was used instead of the "type of antimicrobial" (Table 15, model 2) the "pH" was not an explanatory variable of the HPP inactivation. Probably, the amount of antimicrobial added in the product, especially for those that reduce the pH of the product, explained the same variability of the results as the pH. Thereby, the pH would determine the HPP inactivation of microorganisms due to the acidulation caused by specific antimicrobial compounds rather than the intrinsic pH of RTE meat products.

In the case of a_w, the meta-analysis models 1 and 2 revealed that a_w within the range of 0.870-0.991 was a significant factor (Table 15). Particularly, the factor a_w was found significant when covering an a_w range up to 0.95, but not for the subset of data covering higher a_w values (Table 15, models 3, 4 and 5). These results

suggest a_w of 0.95 as a threshold from which the piezo-protective effect exerted by a_w is relevant and thus the HPP would be significantly less effective. In accordance, results from Article 10 showed that the impact of pressure level and fat content on the behaviour of *L. monocytogenes* and *Salmonella* in dry-cured ham after HPP was significant at $a_w \geq 0.95$.

The piezo-protective effect of a_w has been reported to be dependent of the solute responsible for the low a_w (Sevenich et al., 2015). The application of HPP triggers changes in the molecular responses that are major mechanisms for the stabilization of proteins including the modification of hydrophobic interactions (such as van der Waals interaction), hydrogen-bonding between amino acid residues, electrostatic interactions, and the entropic effect in protein conformation (Mozhaev et al., 1994). Moreover, Miyawaki et al. (2014; 2016) found that the effect of co-solutes on protein stabilization was primarily dependent on a_w, with decreasing a_w values (from 1 to 0.95) increasing the effect of some co-solutes on protein stabilization and working through the cooperative hydration effect. Thereby, one plausible hypothesis for the piezo-protective effect exerted by low a_w could be the stabilization of proteins against the denaturation caused by high pressure.

5.3.1.4. Behaviour of L. monocytogenes and Salmonella in RTE meat products during storage after HPP

In the case of ***L. monocytogenes***, results from Articles 6 and 8 showed that *L. monocytogenes* surviving the HPP treatment (600 MPa/3 min) was able to grow in cooked ham under refrigeration. Compared with control (non-pressurised), after HPP the growth of *L. monocytogenes* showed slightly longer lag times but also a slightly increased growth rate.
Despite the different set up of experiments in terms of type of cooked ham and storage temperature applied in Article 6 (*ad-hoc* manufactured product stored between 8 to 20 °C) and 8 (four commercial products stored at 6 °C), pair-wise comparison of the observed growth rates for non-pressurised and pressurised *L. monocytogenes* confirmed the statistical significance ($p=0.016$) of the differences.
The faster growth of pressurised *L. monocytogenes* indicates a certain piezo-stimulation effect, which could not be explained by any difference or change on the physico-chemical characteristics (such as the pH, a_w, etc.) of the products as the same batch of cooked ham was used with or without HPP and no changes in pH of samples was recoded after HPP.

In the non-growing conditions of dry-cured ham (i.e., $a_w<0.95$), *L. monocytogenes* surviving HPP tended to die (inactivate) during the subsequent refrigerated storage. In matrixes with a_w 0.95 (but not at lower a_w), this inactivation was significantly reduced by increasing the fat content, while it was enhanced by increasing pressure. In conditions allowing the growth of the pathogen (i.e., artificially adjusted at $a_w > 0.95$ according to the CCD of Article 10), the increase in the a_w from 0.96 to 0.98 shortened the lag time and did not change the growth rate of *L. monocytogenes* in dry-cured ham pressurized at 300-450 MPa (Article 10). The behaviour of the pathogen during the storage of dry-cured ham pressurized at 600-750 MPa was very variable, growth occurring only in some samples. The resulting scattered cloud of data points did not allow an accurate estimation of the growth kinetic parameters, presumably due to the higher injury suffered by *L. monocytogenes* cells due to the high intensity HPP applied.

The results of this study provide evidence that pressurized dry-cured ham with a_w values up to 0.96 does not support the growth of *L. monocytogenes* during shelf-life despite this a_w value is notably higher than the value of 0.92 set by the Regulation (CE) 2073/2005 to automatically consider a RTE product within the category 1.3 (not supporting the pathogen growth). Moreover, the identified a_w threshold value (0.96) gives a wide margin of safety to cover the a_w range found in commercial vacuum packaged sliced dry-cured ham, which varied from 0.885 to >0.950, with the median at 0.92 (Hereu, 2014).

Overall, results showed that HPP can be applied as a post-lethality strategy to mitigate the risk of *L. monocytogenes* in RTE meat products by inactivating the pathogen. However, results also pointed out that, due to the potential occurrence of survivors, it would be interesting to combine it with strategies aimed to limit the growth of the pathogen during the subsequent storage after HPP. Strategies combining post-lethality treatments and antimicrobial agents are recognised by public health authorities to potentially achieve synergistic effects and are considered the safest operating procedures for RTE food production, falling into "Alternative 1" of the US Listeria rule (FSIS, 2014) and the recommendations of the Canada Listeria policy (Health Canada, 2011).

In the case of ***Salmonella***, results from Article 1 showed that in RTE meat matrices with high a_w and high pH, such as raw pet food, the storage for 24h under refrigeration at 4 °C (which is below the minimum growth temperature for *Salmonella*) allowed the recovery of HPP damaged *Salmonella* cells, increasing the counts up to 2 log units compared with the samples analysed immediately after HPP. Therefore, the efficacy of HPP as a lethal treatment could be highly overestimated if the enumeration of the pathogen was performed immediately after HPP. Accordingly, the meta-analysis showed that the potential recovery of *Salmonella* after HPP in raw pet food may be significant since the "enumeration time" was found to significantly affect the inactivation (log N/N_0) of microorganisms by HPP (Table 15). In this framework and as reported in Article 3, freezing (-18 °C) could be one possible intervention strategy to minimize the recovery of *Salmonella* after HPP.
On the other hand, results reported in Article 10 showed that the refrigeration temperature (7°C) was the main factor determining the no growth of *Salmonella* in dry-cured ham after HPP. The recovery of *Salmonella* after HPP during the storage of dry-cured ham at 7 °C did not occur. In fact, the refrigerated storage favoured a progressive inactivation of *Salmonella* along the storage time. The a_w of dry-cured ham determined the impact of fat content and pressure level on the inactivation of *Salmonella* during storage after HPP. In this respect, the increase in fat content and pressure level significantly increased *Salmonella* inactivation during storage at a_w >0.95 but not at lower a_w values.

Overall, results showed that HPP can be applied as an intervention strategy to mitigate the risk of *Salmonella* in raw and dry-cured meat products. However, results also pointed out the need to evaluate and apply strategies to avoid the potential recovery of *Salmonella* after HPP in raw meat products as well as to apply strategies to enhance the inactivation of the pathogen during the storage of cured meat products.

5.3.2 Combined effect of antimicrobial and post-lethality strategies on *L. monocytogenes*

In this section, the efficacy of HPP in products formulated with organic acids (lactic acid) and their salts (lactate, diacetate and/or their combination) as antimicrobials and MAP containing CO_2 is discussed.

5.3.2.1. Combination of HPP and organic acids

Lethality of *L. monocytogenes* and *Salmonella* by HPP

As mentioned above, the effect of pH on HPP inactivation of *Salmonella* and *L. monocytogenes* was related to the type and amount of antimicrobial (Table 15). Results provided scientific evidence that, according to the hurdle technology principle, the application of HPP in RTE raw and cooked meat products formulated with lactic acid or diacetate contributed to enhance *Salmonella* and *L. monocytogenes* inactivation compared to products without these antimicrobials.

In this framework, results obtained in different studies (Articles 1-3) showed that the inactivation of *Salmonella* by HPP (450-750 MPa) in raw pet food was enhanced with a lineal (raw pet food for dog) and quadratic effect (raw pet food for cat) in product acidulated with lactic acid (1.5-7.2 g/kg) compared to non-acidulated (Figure 16). The interactions between the different factors evaluated (pressure, holding time and lactic acid) makes it difficult to determine the efficacy of a particular HPP treatment to control *Salmonella* in raw pet food. In this respect, the applied modelling approach allowed to build a decision support tool to quantify the inactivation of *Salmonella* as a function of the pressure, holding time and lactic acid. This decision support tool can be used by food business operators to manage the safety of their products by (i) evaluating the efficacy of given pre-defined processing parameters (lactic acid concentration, pressure and time) and/or (ii) set the combination of processing parameters required to achieve the given pre-defined performance criteria.

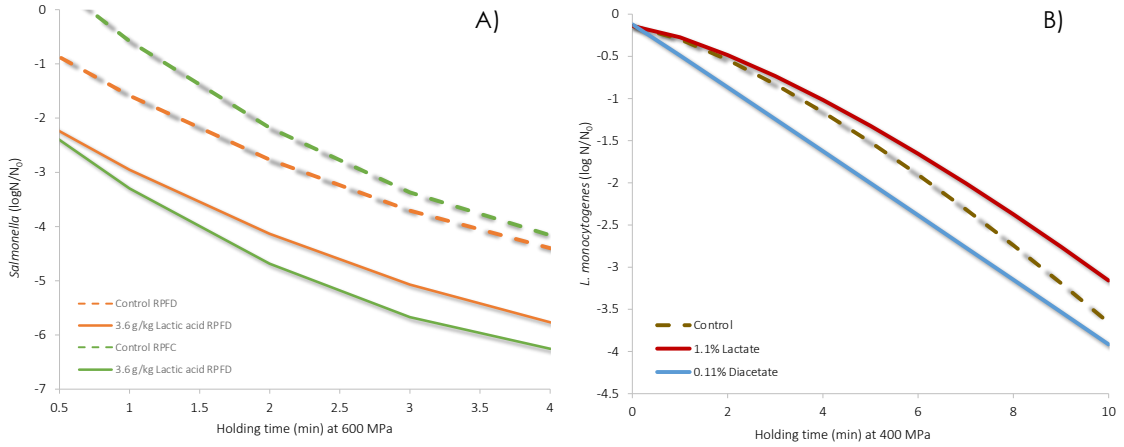


Figure 16. HPP inactivation kinetics predicted by the developed models for (A) *Salmonella* in non-acidulated and acidulated raw pet food for dog (RPFD) and for cat (RPFC) (at 600 MPa, Article 2 and 3) and (B) for *L. monocytogenes* CTC1034 in cooked ham without and with organic acids (at 400 MPa, Article 4).

On the other hand, in **cooked ham,** results reported in Article 4 quantified that the addition of diacetate (0.11%) did not decrease the pH of cooked ham but enhanced the inactivation of *L. monocytogenes* by HPP at 400 MPa compared to cooked ham without organic acids (Figure 16). However, the addition of potassium lactate (with no significant impact on product pH) reduced the inactivation of *L. monocytogenes* by HPP at 400 MPa (piezo-protective effect) compared to cooked ham without antimicrobials (Figure 16). Moreover, the piezo-protective effect exerted by lactate was enhanced by increasing the amount of lactate, i.e., in a dose-dependent manner. Interestingly, this piezo-protective effect was observed for the 3 different *L. monocytogenes* strains evaluated (CTC1034, CTC1011 and Scott A) suggesting that this effect could be not strain specific.

The organic acids (and their salts) can diffuse across the membrane when they are non-dissociated (uncharged protonated form), resulting in an acidification of the bacterial cell cytoplasm as a way to exert their antimicrobial effects (Hirshfield et al., 2003). However, the antimicrobial effect of organic acids depends on the type (e.g. pK_a value), the concentration, the pH of the matrix (determining the amount of undissociated form) and other factors including a_w and temperature among others. Results reported in Article 4 pointed out that lactate acts with different mechanisms on *L. monocytogenes,* leading to a high resistance of the pathogen towards HPP (piezo-protection), compared to lactic acid and diacetate that reduced the HPP resistance.

In order to find out the potential molecular mechanisms involved on the protective effect of lactate on the HPP resistance of *L. monocytogenes*, transcriptomic approaches were explored in the study published in Article 5.

At the gene expression level, the response of *L. monocytogenes* to lactate exposure, even for a short period of time (e.g., from product contamination to HPP), promoted a shift in the central metabolism of the pathogen, favouring the metabolism of 1,2 propanediol and ethanolamine (Article 5). Previous studies have reported that the use of 1,2 propanediol and ethanolamine as a carbon source provided advantage to *L. monocytogenes* to grow in vacuum-packed smoked salmon (Tang et al., 2015) or in co-culture with other bacteria (Anast & Schmitz-Esser, 2020). Thereby, a plausible hypothesis was that the enhanced metabolism of 1,2 propanediol and ethanolamine in response to lactate could also provide some fitness advantage to *L. monocytogenes* to better withstand HPP.

In addition, lactate upregulated genes involved in the methionine synthesis, favouring an activated methyl cycle. Some authors have reported that methyl cycle has a role as endogenous antioxidant and it is involved in membrane lipid biosynthesis (Aktas et al., 2011). Therefore, the activation of methyl cycle could have an impact on *L. monocytogenes* membrane functionality (membrane composition, fluidity and/or integrity) that could presumably confer an enhanced resistance to *L. monocytogenes* towards HPP. Interestingly, acetate was identified to specifically inhibit the synthesis of methionine in *Escherichia coli* with consequently favouring the accumulation of the toxic compound homocysteine (Roe et al., 2002). These results point out that methyl cycle has a relevant role on the inactivation of *L. monocytogenes* by HPP since the piezo-protective effect was exerted by lactate (which activated methyl cycle) but not diacetate (as shown in Article 4).

Behaviour of *L. monocytogenes* and *Salmonella* in RTE meat products during the storage after HPP

The acidulation of **raw pet food** with lactic acid limited the recovery of *Salmonella* after HPP during the subsequent storage at 4 °C for 24h, and higher HPP inactivation was recorded compared to non-acidulated raw pet food (Article 1). Moreover, results showed that the implementation of a freezing storage at -18 °C for 2 weeks after HPP favoured further inactivation of the pathogen in the acidulated product (Article 3). Thus, results proved that intervention strategies involving the application of HPP, acidulation with lactic acid and the implementation of a subsequent freezing storage can be combined to minimize the risk of *Salmonella* recovery after HPP and to enhance its inactivation in raw pet food (Articles 1-3).

In **cooked ham**, the application of HPP (600 MPa, 3 min) extended the safe shelf-life of products by 2 to 2.75- fold compared to non-pressurized cooked ham when no organic acids were used in the formulation (Table 16, cases 1, 4 and 7). The addition of lactate enhanced the piezo-stimulation exerted by HPP on *L. monocytogenes* growth, which was able to grow in HPP products formulated with organic acids at concentrations that prevented growth in non-pressurised cooked ham (section 5.2, Article 6). For instance, as shown in Table 16, in cooked ham formulated with 2% lactate and 0.11% diacetate stored at 8 °C, *L. monocytogenes* was able to grow after HPP, while tended to inactivate in non-HPP conditions (case 3). Therefore, the piezo-stimulation effect moved the growth/no growth boundary of *L. monocytogenes* and higher concentrations would be needed to inhibit its growth. In this respect, in cooked hams formulated with 2% lactate and 0.45% diacetate, the inhibition effect of diacetate on *L. monocytogenes* growth prevailed over the piezo-stimulation effect of lactate, resulting in the progressive inactivation of the pathogen along storage (cases 6 and 11).

The growth rates of the pathogen in pressurised products with lactate were markedly higher than for non-pressurised products with the same concentrations of organic acids. This was observed at all tested storage temperatures (8 - 20 °C), being particularly pronounced at 20 °C, with a *L. monocytogenes* growth 4-fold faster in products with lactate (Article 6). Although the combination of lactate and HPP allowed the extension of the safe shelf-life compared to the control products (without organic acids non-HPP), the application of HPP in products formulated with lactate shortened the safe shelf-life compared to non-HPP

counterparts (cases 2, 8 and 9). In the same line, the growth of *L. monocytogenes* was also piezo-stimulated in cooked ham formulated with a combination of organic acids including 2% lactate and 0.11% diacetate compared non-HPP conditions (case 10), indicating that the piezo-stimulation effect of lactate prevailed over the presumably inhibition effect of diacetate.

For these safe shelf-life estimations, the piezo-protection effect exerted by lactate could not be quantified because *L. monocytogenes* was not detected in most of the samples immediately after 600 MPa. Therefore, if piezo-protection had occurred, the estimated safe shelf-life of pressurized cooked ham formulated with lactate would be even shorter.

All these results suggest that due to the specific effect of the type and amount of antimicrobials on the HPP inactivation of pathogens and subsequent behaviour, the impact of the formulation of cooked ham should be carefully assessed in order to validate the efficacy of the strategy as control measure within the HACCP plan. In this regard, a future perspective is to assess the impact of the application of HPP combined with organic acids on the probability of illness (listeriosis) due to the consumption of RTE cooked meat products (exposure/risk characterization) through a quantitative microbial risk assessment approach.

Table 16. Impact of HPP (600 MPa for 3 min) on the safe shelf-life in vacuum-packed cooked hams formulated without or with organic acids included in Article 6.

Case	Organic acid	Storage temperature (°C)	Impact of the HPP on the safe shelf-life[a] compared to non-HPP counterpart	Safe shelf-life[a] extension in relation to control products (without organic acids non-HPP)
1	None	8	From 8 to 18 days (x2.3)	From 8 to 18 days (x2.3)
2	2.8% lactate	8	From 61 to 58 days (x0.95)	From 8 to 58 days (x7.3)
3	2% lactate +0.11% diacetate	8	From non-constraining[b] to 59 days (from no-growth to growth domain)	From 8 to 59 days (x7.4)
4	None	12	From 4 to 11 days (x2.75)	From 4 to 11 days (x2.75)
5	4% lactate	12	Remained non-constraining[b]	From 4 to non-constraining[b]
6	2% lactate +0.45% diacetate	12	Remained non-constraining[b]	From 4 to non-constraining[b]
7	None	20	From 2 to 4 days (x2)	From 2 to 4 days (x2)
8	2.8% lactate	20	From 7 to 5 days (x0.71)	From 2 to 5 days (x2.5)
9	4% lactate	20	From 18 to 13 days (x0.72)	From 2 to 13 days (x6.5)
10	2% lactate +0.11% diacetate	20	From 13 to 10 days (x0.77)	From 2 to 10 days (x5)
11	2% lactate +0.45% diacetate	20	Remained non-constraining[b]	From 2 to non-constraining[b]

[a] Safe shelf-life of products estimated as the time needed for *L. monocytogenes* to reach 100 cfu/g considering an initial level of the pathogen of -1.4 log cfu/g.
[b]Shelf-life not dependent on the growth of *L. monocytogenes.*

5.3.2.2. Combination of HPP and MAP

The meta-analysis models of inactivation data showed that, overall, the effect of packaging was not strong enough to be a statistically significant factor on the **HPP inactivation** of *L. monocytogenes* (Table **15**) as reported in Article 7, where the packaging of cooked ham in MAP (20% CO_2: 80% N_2) 1h prior HPP tended to slightly increase the inactivation of *L. monocytogenes* by HPP in all commercial cooked hams compared to vacuum packaging conditions. However, the effect was not statistically significant, and it was neither observed with the longer exposure of *L. monocytogenes* to MAP (packed 24h prior HPP and stored at 4 °C). Little information is available on the literature about the effect of MAP on HPP inactivation of pathogens. In RTE cold-smoked salmon, clear enhancement of the inactivation was observed by Amanatidou et al. (2000) for long treatments and mild pressure treatments (150MPa for 15-30 min) when 50% CO_2: 50% O_2 MAP was compared with vacuum-packaging. For much shorter holding times (0.5 and 1 min) at much higher pressures (500 and 700 MPa), slightly lower counts of inoculated *Listeria innocua* were found when

smoked salmon was pressurised in 50% CO_2: 50% N_2 MAP compared with vacuum-packaging, though the differences were not statistically significant (Hafsteinsson et al. 2007).

Despite the low impact of the packaging system on the immediate HPP inactivation, a beneficial effect of combination was observed during the **storage after HPP**. Results from Article 8 showed that, generally, the combined used of HPP and MAP extended the lag time and lowered the growth rate of *L. monocytogenes* after HPP. In this line, the application of HPP (600 MPa, 3 min) and MAP increased by 2.42 up to 3.64-fold the safe shelf-life of cooked hams compared control (non-pressurized vacuum-packed cooked ham), while the application of only HPP resulted in a lower extension of the safe shelf-life (by 1.76 up to 2.43-fold) of cooked hams under vacuum packaging conditions (Table 17). The final relevance of MAP for products intended to HPP would need further analysis of the cost-risk-benefit ratios, as HPP of MAP products with head space is less efficient than vacuum-packaged products.

Table 17. Impact of HPP (600 MPa for 3 min) on *L. monocytogenes* behaviour in cooked hams included in Article 8 under vacuum and MAP conditions stored at 6 °C.

Cooked ham		Packaging[a]	Impact on the safe shelf-life, days in relation to non-HPP products (extension relative to the control)[b]
Brand	Formulation		
A	Standard	VP	From 25 to 44 days (x1.76)
A	Sodium-reduced	VP	From 18 to 38 days (x2.11)
B	Standard	VP	From 36 to 81 days (x2.25)
B	Sodium-reduced	VP	From 14 to 34 days (x2.43)
A	Standard	MAP	From 38 to 77 days (x3.08)
A	Sodium-reduced	MAP	From 28 to 62 days (x3.44)
B	Standard	MAP	From 40 to 87 days (x2.42)
B	Sodium-reduced	MAP	From 24 to 51 days (x3.64)

[a]VP: vacuum packaging; MAP: modified atmosphere (20% CO_2:80% N_2)
[b]Safe shelf-life of products estimated as the time needed for *L. monocytogenes* to reach 100 cfu/g considering an initial level of the pathogen of -1.4 log cfu/g. In brackets the extension of safe shelf-life compared to control product (vacuum-packed and non-pressurized) thanks to the application of HPP.

5.3.3 Corrective storage to enhance pathogen inactivation in dry-cured and cured-fermented meat products

One of the main drawbacks of HPP is its reduced efficacy at low a_w (EFSA BIOHAZ Panel, 2022), which can lead to a limited efficacy of HPP to control *L. monocytogenes* and *Salmonella* in dry-cured and cured-fermented RTE meat products such as dry-cured ham and dry-fermented sausages. However, alternative intervention strategies to control pathogens in these products can be designed taking advantage of the metabolic exhaustion phenomena associated with the hurdle technology (Leistner and Gorris, 1995).

Specifically, the implementation of a corrective storage before delivering the products to the market was assessed to favour the inactivation of *L. monocytogenes* in dry-cured ham (Article 11) and *Salmonella* in dry-fermented sausages (Article 12). In both studies, the storage of products in the range of temperatures from 4-2 °C to 25 °C showed that the inactivation was linearly enhanced by increasing storage temperature. Moreover, in both studies, products with lower pH (Iberian dry-cured ham and acid dry fermented sausages) favoured the inactivation of pathogens compared to the same products with a higher pH (Serrano dry-cured ham and low-acid dry fermented sausages). However, the role of a_w on pathogen inactivation was different for *L. monocytogenes* and *Salmonella*. While lowering the a_w (from 0.91 to 0.85-

0.87) of dry-cured ham enhanced the inactivation of *L. monocytogenes* (Article 11), the opposite was found for *Salmonella* in dry-fermented sausages with a_w from 0.93 to 0.88 (Article 12). In Article 11, *L. monocytogenes* was inoculated on dry-cured ham slices with different a_w (as final product) immediately before the storage, mimicking the post-processing recontamination during slicing and/or packaging of the product. On the other hand, in Article 12, *Salmonella* was inoculated in the raw meat used in the manufacturing of the dry-fermented sausages, as usually occurs for these products, thus it was submitted to the fermentation and ripening/drying processes before being stored at the different temperatures. In this case, *Salmonella* could develop stress response mechanisms (i.e., adaptation) during the fermentation and ripening/drying processes to withstand a progressive reduction of a_w, which might provide resistance to low a_w values during the storage.

The quantitative approach used to study the impact of the storage temperature and a_w on *L. monocytogenes* and *Salmonella* inactivation in dry-cured ham and dry-fermented sausages allowed to develop a decision support tool that can be used to design a corrective storage to achieve the desired reduction of pathogens according to the performance criteria established in the HACCP (Articles 11 and 12).

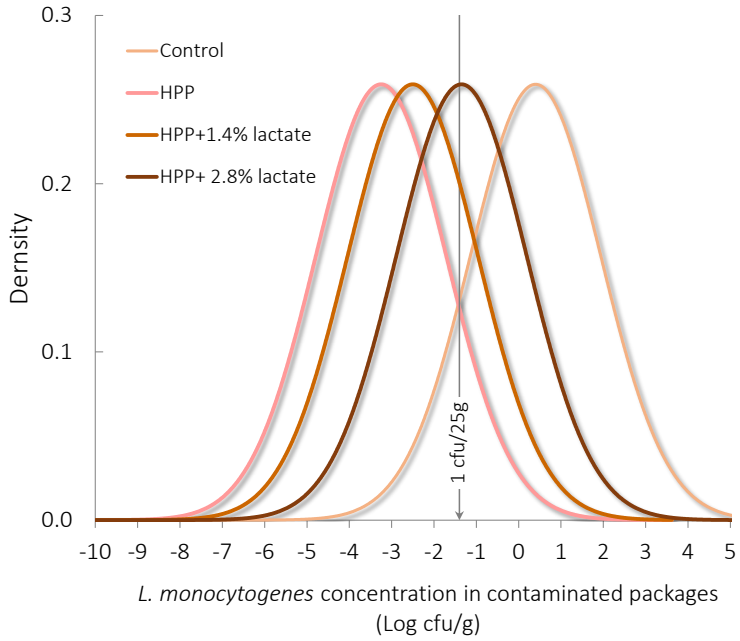
5.4. Contribution of intervention strategies applied on the compliance of performance objective for *L. monocytogenes* and *Salmonella*

In this section, the contribution of HPP and its combination with other intervention strategies on the fulfilment of the performance objective (just before the release of the product to the market) for *L. monocytogenes* and *Salmonella* is assessed.

5.4.1 *Listeria monocytogenes*

Under the hypothetical scenario that a RTE **cooked meat product** is contaminated by *L. monocytogenes*, the initial concentration of the pathogen can be described by a normal distribution as represented in Figure 11 (mean 0.40; standard deviation 1.54, see section 3.2.3). Under this assumption, 88% of the contaminated products would have an analytically detectable concentration of *L. monocytogenes* of at least 1 cfu in 25g (≥-1.4 log cfu/g).

According to the lethality of HPP observed in the studies carried out in this thesis (Article 4), the application of 400 MPa for 10 min in vacuum-packed cooked ham formulated without organic acids reduced to 11.4% the proportion of contaminated products with a detectable concentration of ≥-1.4 log cfu/g. Following the same approach, the piezo-protective effect of lactate and thus the reduction of the HPP efficacy can also be quantified. Thus, for vacuum-packed cooked ham formulated with 1.4 or 2.8% lactate, the proportion of contaminated products with a detectable level of *L. monocytogenes* (≥-1.4 log cfu/g) would be 24% and 51%, respectively (Figure 17).

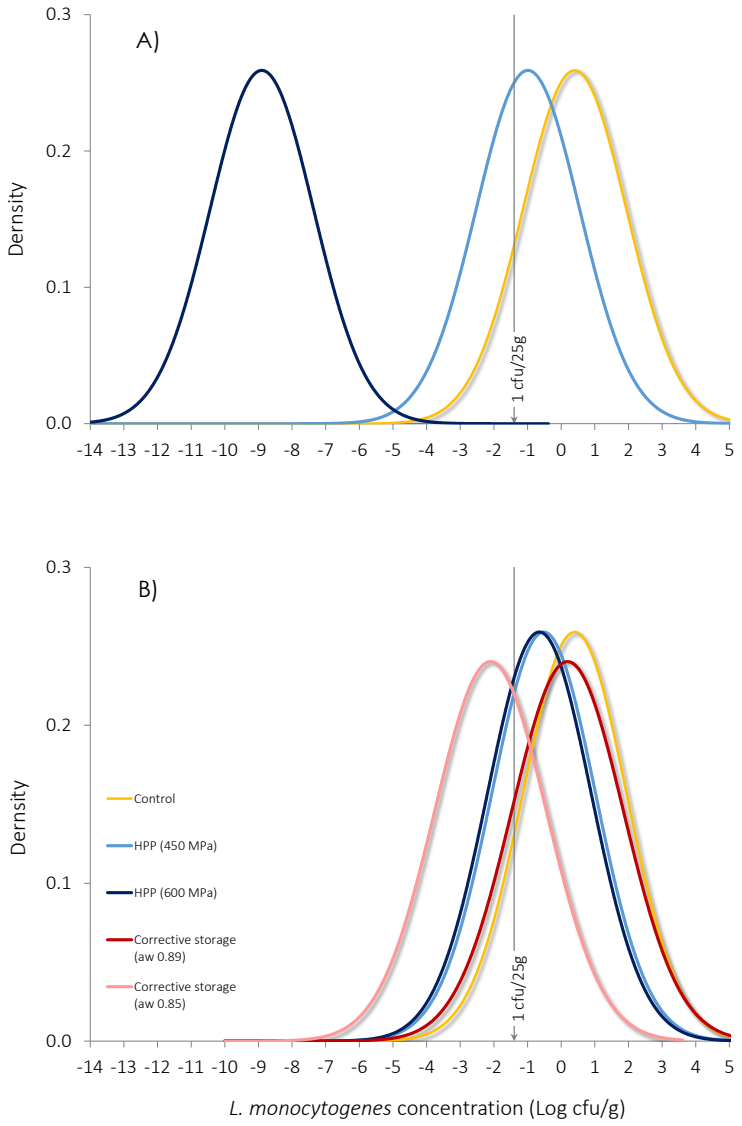


HPP	Lactate (%)	Predicted inactivation (ΣR, log N/N_0)[a]	H_0-ΣR (log cfu/g)	P ($x \geq PO$) (%)	Relative risk (%)
-	0	-	0.40 ± 1.54	88.04	100
400 MPa for 10 min	0	3.66 ± 0.05	-3.25 ± 1.54	11.41	12.91
	1.4	2.91 ± 0.04	-2.50 ± 1.54	23.66	26.76
	2.8	1.76 ± 0.05	-1.35 ± 1.54	51.24	57.96

[a]Mean and standard deviation of the inactivation observed with plate counting for 3 replicates of *L. monocytogenes* in cooked hams used in Article 4.

Figure 17. Normal distributions describing the concentration of *L. monocytogenes* CTC1034 in contaminated cooked ham (see section 3.2.3.) before and after HPP for products without and with lactate and the corresponding percentage of the distribution density above 1 cfu in 25 g.

In the case of **dry-cured meat products**, the impact of HPP was assessed in products with a_w of 0.89 and 0.94 (percentile 5th and 95th of the a_w measured from 62 commercial dry-cured hams (Hereu, 2014). The application of HPP at 450 MPa for 5 min drastically reduced the proportion of contaminated dry-cured hams, indicating that only 2.6% (a_w of 0.94) or 5.1% (a_w of 0.89) of the products would have a *L. monocytogenes* concentration ≥1.4 log cfu/g (Figure 18). When the HPP applied was more intense (600 MPa for 5 min), results showed that the proportion of dry-cured hams (a_w of 0.94) contaminated with detectable concentrations of *L. monocytogenes* was extremely low (less than 1 out of 10^{10}) (Figure 18). However, when the same HPP treatment was applied in dry-cured hams with a_w of 0.89, the piezo-protection of the aw did not result in a relevant improvement of the HPP efficacy compared to HPP at 450 MPa (Figure 18). These results highlighted the impact of the piezo-protective effect of low a_w on *L. monocytogenes* inactivation by HPP. In this framework, to enhance *L. monocytogenes* inactivation in dry-cured ham with low a_w, the implementation of a corrective storage before commercialization as a control measure was assessed. Results showed that the application of a corrective storage at 22 °C for 7 days reduced by 82-86% the proportion of contaminated Iberian and Serrano dry-cured hams with a_w of 0.89 indicating that 13-14% products would have a *L. monocytogenes* concentration of ≥1.4 log cfu/g (Figure 18). The efficacy of the corrective storage was enhanced in dry-cured hams with low a_w (0.85-0.87), where the application of this control measure reduced to 91-99% the proportion of contaminated dry-cured hams, i.e., only 1-9% of the products having detectable levels of *L. monocytogenes*.



Control measure	a_w	Inactivation (ΣR, log N/N$_0$)	H$_0$-ΣR (log cfu/g)	P ($x \geq$ PO) (%)	Relative risk (%)
-		-	0.40 ± 1.54	88.04	100
HPP at 450 MPa for 5 min	0.94 (30% fat)	1.39 ± 0.02[a]	-0.99 ± 1.54	2.61	2.96
	0.89 (18% fat)	0.92 ± 0.05[a]	-0.52 ± 1.54	5.10	5.79
HPP at 600 MPa for 5 min	0.94 (30% fat)	6.40 ± 0.00[ab]	-8.90 ± 1.54	$7.32 \cdot 10^{-11}$	$8.31 \cdot 10^{-11}$
	0.89 (42% fat)	1.05 ± 0.06[a]	-0.65 ± 1.54	4.28	4.86
Corrective storage at 22 °C for 7 days	0.89 (Iberian)	0.21 ± 0.62[c]	0.19 ± 1.66	13.78	17.65
	0.85 (Iberian)	2.50 ± 0.62[c]	-2.10 ± 1.66	0.68	0.77

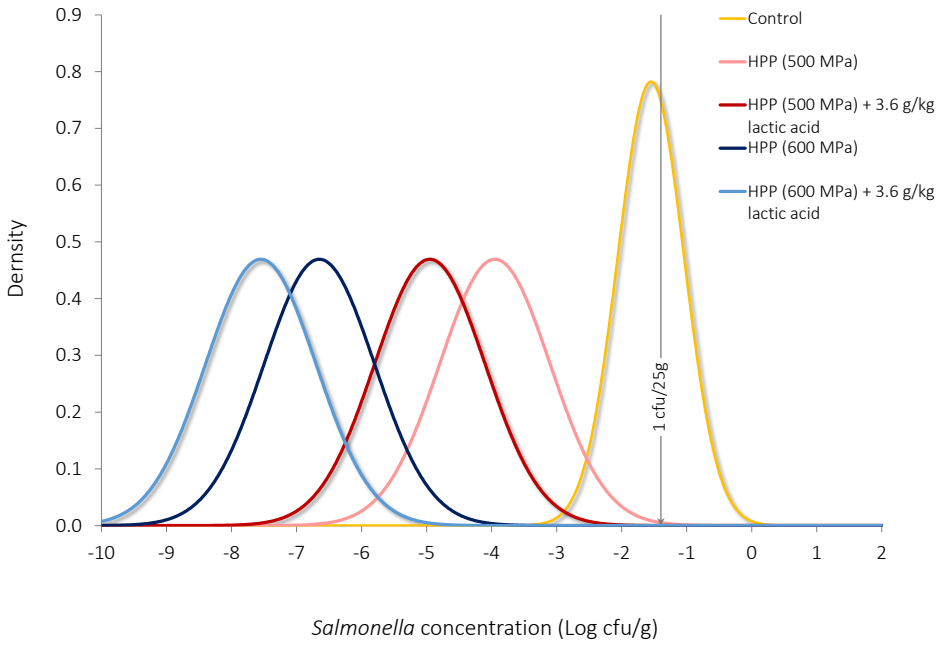
[a]Mean and standard deviation of the inactivation observed with plate counting for 3 replicates of *L. monocytogenes* in dry-cured ham used in Article 10.
[b]*L. monocytogenes* concentration after HPP was below the detection limit (4 cfu/g). It was assumed that the minimum inactivation was the difference between inoculum (7 log cfu/g) and detection level (4 cfu/g).
[c]Inactivation of *L. monocytogenes* predicted with model developed in Article 11.

Figure 18. Normal distributions describing the concentration of *L. monocytogenes* in vacuum packed dry-cured ham with a_w 0.94 (a) and $a_w \leq 0.89$ (b) submitted to HPP or to a corrective storage period and the corresponding percentage of the distribution density above 1 cfu in 25 g.

5.4.2 *Salmonella*

For **raw pet food** without any specific control measure (control scenario), the analysis derived from the presence of *Salmonella* in production lots (including products negative and positive for *Salmonella*) showed that the concentration of the pathogen in raw pet food could be described with a normal distribution (mean -1.55 log cfu/g; standard deviation 0.51 log cfu/g) (Article 2). According to this distribution, 38% of the products could be contaminated with a concentration of *Salmonella* of at least 1 cfu in 25g (≥ -1.40 log cfu/g) as shown in Figure 19.

To increase the safety of raw pet food and to accomplish with zero tolerance policies for *Salmonella*, HPP can be implemented as kill step (control measure). In this framework, the microbial inactivation achieved with 500 MPa for 5 min in non-acidulated raw pet food, shifted the distribution of *Salmonella* concentration to the left, with 0.13-0.14% the products with ≥ -1.40 log cfu/g. When HPP is applied in raw pet food acidulated with 3.6 g/kg lactic acid, less than 0.001% of the products would be contaminated with a *Salmonella* concentration ≥ -1.40 log cfu/g. In case there is no interest in adding lactic acid (a preservative with E code) in the formulation aiming at clean labelling, HPP intensity can be increased up to 600 MPa for 5 min to get equivalent results, i.e. 0.001% of products contaminated with a detectable *Salmonella* concentration (Figure 19).



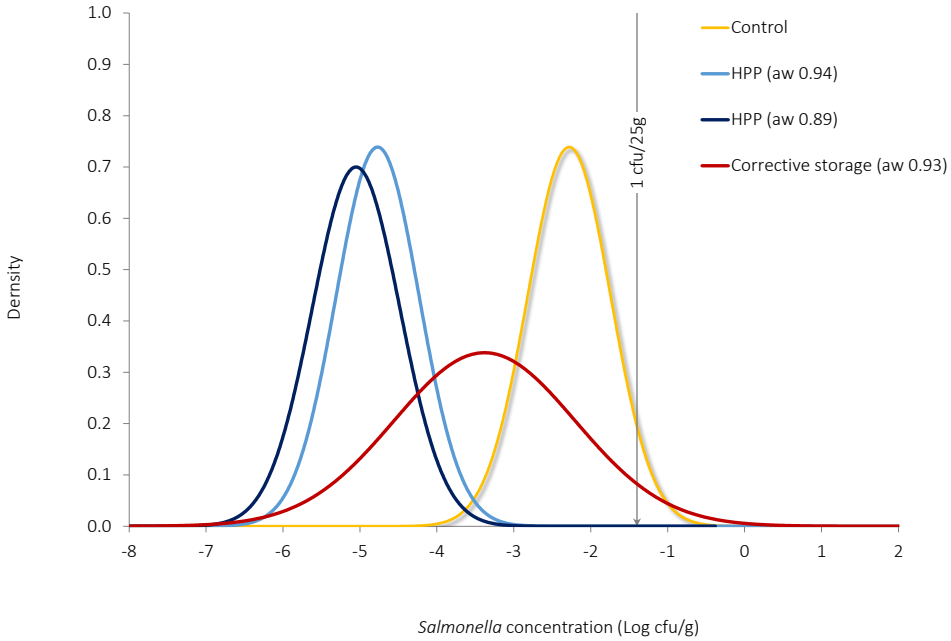
HPP	Lactic acid (g/kg)	Inactivation (ΣR, log N/N_0)[a]	H_0-ΣR (log cfu/g)	P ($x \geq PO$) %	Relative risk (%)
-	0	-	-1.55 ± 0.51	38.44	100
500 MPa for 5 min	0	2.40 ± 0.68	-3.95 ± 0.85	0.13	0.34
	3.6	3.40 ± 0.68	-4.95 ± 0.85	$1.48 \cdot 10^{-3}$	$3.85 \cdot 10^{-3}$
600 MPa for 5 min	0	5.10 ± 0.68	-6.65 ± 0.85	$3.28 \cdot 10^{-8}$	$8.53 \cdot 10^{-8}$
	3.6	6.00 ± 0.68	-7.55 ± 0.85	$2.32 \cdot 10^{-11}$	$6.04 \cdot 10^{-11}$

[a]Inactivation of *Salmonella* in raw pet food for dog (RPFD) predicted through models developed in Article 2.

Figure 19. Normal distributions describing the concentration of *Salmonella* in non-acidulated and acidulated raw pet food for dog submitted to HPP of 500 or 600 MPa for 5 min and the corresponding percentage of the distribution density above 1 cfu in 25 g.

In the case of RTE **dry-fermented sausages**, according to the data available regarding the occurrence of *Salmonella* (Table 11), the concentration would be normally distributed with a mean of -2.28 log cfu/g and a standard deviation 0.54, with 5.16% of the products ≥ -1.40 log cfu/g (Figure 20).

To enhance *Salmonella* inactivation in dry cured and fermented meat products, HPP or corrective storage can be applied as control measures. The efficacy of these intervention strategies was assessed for products with a_w values of a_w 0.89 and 0.94 (percentiles 5th and 95th of the a_w measured from 62 commercial dry-cured hams) (Hereu, 2014). The lethality caused by HPP at 450 MPa for 5 min reduced to practically zero the proportion of products with a *Salmonella* concentration ≥ -1.40 log cfu/g (regardless of their a_w). On the other hand, the application of a corrective storage at 22 °C for 7 days reduced ca. 1 log the level of pathogen, but a high standard deviation (1.18 log cfu/g) was associated with this effect, which implies that 4.76% of the density distribution for *Salmonella* concentration is at ≥ -1.40 log cfu/g.



Control measure	a_w	Inactivation (ΣR, log N/N$_0$)	H$_0$-ΣR (log cfu/g)	P (x ≥ PO) %	Relative risk %
-		-	-2.28 ± 0.54	5.16	100
HPP at 450 MPa for 5min	0.94 (30% fat)	2.49 ± 0.03[a]	-4.77 ± 0.54	$2.32 \cdot 10^{-8}$	$4.5 \cdot 10^{-7}$
	0.89 (18% fat)	2.77 ± 0.17[a]	-5.05 ± 0.57	$5.69 \cdot 10^{-9}$	$1.1 \cdot 10^{-7}$
Corrective storage at 22 °C for 7 days (acid-type)	0.93 (acid-type)	1.09 ± 1.05[b]	-3.37 ± 1.18	4.76	0.92

[a] Mean and standard deviation of the inactivation observed with plate counting for 3 replicates of *Salmonella* in dry-cured ham used in Article 10.
[b] Inactivation of *Salmonella* predicted with model developed in Article 12.

Figure 20. Normal distributions describing the concentration of *Salmonella* in dry-cured ham with a_w 0.94 and a_w 0.89 submitted to HPP or in dry-fermented sausages submitted to a corrective storage period and the corresponding percentage of the distribution density above 1 cfu in 25 g.

CONCLUSIONS

6. Conclusions

In agreement with the objectives of this PhD, the results obtained in the included studies and globally discussed in the present document, the following conclusions can be drawn:

1. According to the gamma concept modelling approach, storage temperature constitutes the factor with the major impact on ***L. monocytogenes*** and ***Salmonella*** growth, both in terms of probability and rate, in most RTE meat products. In **raw and cooked meat products** the high growth probability (>90%) of both pathogens, makes it highly relevant the identification, application and validation of intervention strategies based on antimicrobial agents to inhibit the growth and/or post-lethality treatments to decrease the level of pathogens. In **dry-cured and dry-fermented meat products**, growth/no growth boundary of *L. monocytogenes* and *Salmonella* was found at a_w values of 0.95-0.96. However, the survival of both pathogens under non-growing conditions motivates the interest in intervention strategies aiming to reduce the level of pathogens.

2. The impact of **antimicrobial strategies** such as biopreservation, i.e., organic acids and bioprotective culture as well as modified atmosphere packaging (MAP) on *L. monocytogenes* growth inhibition and/or safe shelf-life extension of cooked ham depends on the type and/or intensity of the intervention strategy applied. **Lactate and diacetate**, at concentrations usually used by meat industry, increase the lag time and decrease the growth rate of *L. monocytogenes* resulting in a safe shelf-life that can at least treble that of the control without organic acids. For **MAP**, the safe shelf-life extension would be less than the double. **Bioprotective strain** *Latilactobacillus sakei* CTC494 reduces the maximum population density of *L. monocytogenes* and can totally inhibit its growth at non-isothermal storage conditions ranging from 2 to 8 °C.

3. The strategy of "**safety by design**" using predictive modelling is proved to be a suitable strategy to formulate RTE cooked ham not supporting the growth of *L. monocytogenes*. The ability of *L. monocytogenes* CTC1034 strain to grow faster in RTE cooked meats compared to EUR-Lm reference and clinical strains makes it a suitable strain to be used in validation studies aiming to determine the safe shelf-life of RTE cooked meats covering worst-case scenarios.

4. The impact of **high pressure processing** (HPP) as post-lethality strategy is dependent on the microbial **strain** and its **physiological state**, requiring the selection of specific strains and specific protocols for inoculum preparation when assessing its efficacy. In this PhD, a versatile pool of *L. monocytogenes* strains (CTC1034, CTC1011 and Scott A) with different HPP inactivation curves and a pool of HPP resistant *Salmonella* strains (CTC1022, GN0082 and GN0085) were established for cooked ham and raw pet food, respectively, to conduct HPP validation studies through challenge testing. Cultures grown at 37 °C without or with subsequent freezing at -80 °C would be recommended for HPP validation studies due to their higher piezo-resistance compared to the cold-adapted strains recommended for safe shelf-life validation studies.

5. **HPP parameters** determine the magnitude of pathogen inactivation. Generally, with increasing pressure and holding time, a higher inactivation of pathogens is found, though not always with linear kinetics. For instance, **resistance tails** can result in survivors that in cooked ham can grow with a piezo-stimulated rate during subsequent storage.

6. The efficacy of HPP depends on RTE product, being **a_w** a key intrinsic parameter for *L. monocytogenes* and *Salmonella* inactivation by HPP. A piezo-protective effect of a_w significantly occurs at a_w values below of 0.95. Moreover, a_w determines the impact of fat and pressure level on the behaviour of *L. monocytogenes* and *Salmonella* after HPP in dry-cured ham.

7. The **combination of strategies** does not always result in a beneficial interaction as could be expected within the hurdle technology concept. **Lactate** exerts a dose-dependent **piezo-protective effect** on the inactivation of *L. monocytogenes* by HPP. The **upregulation of genes** involved in 1,2 propanediol and ethanolamine metabolism and methionine synthesis in the presence of lactate could hypothetically provide fitness advantage to the pathogen to withstand HPP. Additionally, lactate enhances the **piezo-stimulation** of the growth during the subsequent storage. As a consequence, the safety improvement and extension of the safe shelf-life is smaller than when HPP and lactate are applied as single strategies.

8. Beneficial interactions result from the combination of HPP and **diacetate** in cooked ham, enhancing *L. monocytogenes* immediate inactivation and slowing down its subsequent growth. In cooked ham, **MAP** does not increase *L. monocytogenes* immediate inactivation by HPP but slows down its subsequent growth. On the other hand, acidulation of raw pet food with **lactic acid** enhances the *Salmonella* inactivation by HPP, increasing the inactivation rate and, as also achieved with the corrective storage at -18 °C, avoiding the recovery of sublethally injured cells.

9. In RTE meat products not supporting the growth of *L. monocytogenes* and *Salmonella*, **corrective storage** taking advantage of product characteristics and metabolic exhaustion mechanisms can be another post-processing strategy to inactivate pathogens. The efficacy of corrective storage was enhanced by increasing storage temperature and in products with lower pH. Regarding the impact of a_w, for *L. monocytogenes* in dry-cured ham the lower the a_w the higher the inactivation. The contrary applies for *Salmonella* in dry-fermented sausages, presumably related to the resistance acquired during the fermentation and ripening processes.

10. **Decision support tools** based on predictive models developed in this PhD will help stakeholders with the assessment and application of the studied intervention strategies as control measures for *L. monocytogenes* and *Salmonella* in RTE meat products through a product-oriented approach. They can be used to set the conditions and/or technological parameters to achieve the desired reduction of the pathogens and/or the expected safe shelf-life. Ultimately, these predictive tools offer an effective means of validation of control measures within the HACCP plan.

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