UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE FARMACIA

TESIS DOCTORAL

Listeria monocytogenes en productos cárnicos curados: incidencia, supervivencia y control mediante tratamientos de altas presiones y bioconservación

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Aida Perez Baltar

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AIDA PÉREZ BALTAR

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"Y la vida siguió como siguen las cosas que no tienen mucho sentido"

J. Sabina

Serendipia

(noun) casualidad, fortuna

Descubrimiento o hallazgo afortunado, valioso e inesperado que se produce de manera accidental, casual o por destino, o cuando se está buscando una cosa distinta.

A mi madre

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Lista de abreviaturas y acrónimos

a*	Tendencia al rojo en el sistema de color CIELab
ABC	*ATP binding cassette* (dominio de unión a ATP)
ADN	Ácido desoxirribonucleico
ADNc	ADN complementario
ARN	Ácido ribonucleico
ARNm	ARN mensajero
ARNr	ARN ribosómico
AEs	Aceites esenciales
ANOVA	*Analysis of variance* (análisis de varianza)
APH	Altas presiones hidrostáticas
APPCC	Análisis de Peligros y Puntos de Control Crítico
ATP	Adenosín trifosfato
a_w	Actividad de agua
BHI	*Brain heart infusion* (infusión de cerebro y corazón)
CB	Cloruro de benzalconio
CDC	*Centers for Disease Control and Prevention* (Centros de Control y Prevención de Enfermedades)
CE	Comisión Europea
CMI	Concentración mínima inhibitoria
C_T	*Threshold cycle* (ciclo umbral)
kb	Kilobase
kDa	Kilodalton
kGy	Kilogray
ECDC	*European Centre for Disease Prevention and Control* (Centro Europeo para Prevención y Control de Enfermedades)
EE. UU.	Estados Unidos
EFSA	*European Food Safety Authority* (Autoridad Europea de Seguridad Alimentaria)
FDA	*Food and Drug Administration* (Administración de Alimentos y Medicamentos)

FSIS	*Food Safety and Inspection Service* (Servicio de Inspección y Seguridad Alimentaria)
FSO	*Food Safety Objective* (Objetivo de Seguridad Alimentaria)
GRAS	*Generally recognized as safe* (reconocidos generalmente como seguros)
IGS	*Intergenic spacer* (región intergénica espaciadora)
L*	Luminosidad en el sistema de color CIELab
LIPI	Listeria *pathogenicity island* (isla de patogenicidad de *Listeria*)
MLST	*Multilocus sequence typing* (tipado de secuencias de múltiples genes)
MLVA	*Multiple-locus variable number of tandem repeat analysis* (análisis del número variable de repeticiones en tándem)
MOPS-BLEB	*Morpholinepropanesulfonic acid-buffered* Listeria *enrichment broth* (caldo de enriquecimiento de *Listeria* tamponado con ácido morfolinpropanosulfónico)
MOX	*Modified Oxford agar* (agar Oxford modificado)
MPa	Megapascales
MVLST	*Multi-virulence-locus sequence typing* (tipado de secuencias de múltiples genes de virulencia)
PCR	*Polymerase chain reaction* (reacción en cadena de la polimerasa)
PFGE	*Pulsed-field gel electrophoresis* (electroforesis en gel en campo pulsante)
PMSCs	*Premature stop codons* (codones de parada prematuros)
qPCR	*Quantitative PCR* (PCR cuantitativa)
QPS	*Qualified presumption of safety* (presunción cualificada de seguridad)
RT-qPCR	*Quantitative real-time PCR* (PCR cuantitativa en tiempo real)
RTE	*Ready-to-eat* (listo para el consumo)
SNP	*Single nucleotide polymorphism* (polimorfismo de un único nucleótido)
SSI-2	*Stress survival islet 2* (isleta de supervivencia a estrés 2)
ST	*Sequence type* (tipo de secuencia)
TVC	*Total viable counts* (recuento de totales viables)
UE	Unión Europea
UFC	Unidades formadoras de colonias

USDA	*United States Department of Agriculture* (Departamento de Agricultura de EE. UU.)
UVM	*University of Vermont modified* (Universidad de Vermont modificado)
VBNC	*Viable but nonculturable state* (estado viable pero no cultivable)
WGS	*Whole-genome sequencing* (secuenciación del genoma completo)

Resumen

Listeria monocytogenes es una bacteria patógena causante de listeriosis, una enfermedad que se produce principalmente por la ingesta de alimentos listos para el consumo (RTE) contaminados, siendo los productos cárnicos los asociados con mayor frecuencia a casos de listeriosis. El jamón curado es un producto cárnico RTE curado-madurado de gran aceptación en el mercado español y cuya exportación a otros países se ha incrementado notablemente en los últimos años. Se considera un producto seguro por su baja actividad de agua (a_w) y alta concentración de sal, pero puede contaminarse con *L. monocytogenes* durante las operaciones de deshuesado y loncheado previas a su comercialización. La legislación europea permite hasta 100 UFC/g de *L monocytogenes* en productos que no puedan soportar su crecimiento o que no se destinen a fines médicos o alimentación infantil, mientras que otros países, como EE. UU., aplican políticas más restrictivas de tolerancia cero o ausencia en 25 g de producto. En la presente tesis doctoral se plantearon los siguientes objetivos: caracterizar la presencia de *L. monocytogenes* en las zonas de deshuesado y loncheado de industrias productoras de jamón curado, aplicar tratamientos de altas presiones, bioconservación y sus combinaciones para inactivar *L. monocytogenes* en jamón curado, y, por último, investigar si estos tratamientos afectan a la expresión de genes de resistencia a estrés y/o virulencia de las células del patógeno supervivientes.

Se aisló *L. monocytogenes* en el 11,8% de las muestras de ambiente y superficies de las zonas de deshuesado de tres industrias productoras de jamón curado. La mayoría de las muestras positivas procedían de zonas de no contacto con el jamón, recogidas durante el procesado, aunque el patógeno también se aisló tras la limpieza y desinfección de las superficies y ambiente industrial. El serotipo 1/2a fue el más abundante, seguido de los serotipos 1/2b y 1/2c, mientras que solo se registró un aislado del serotipo 4b. Se identificaron 20 pulsotipos diferentes, cuatro de los cuales se consideraron persistentes. Los aislados obtenidos se agruparon en 11 tipos de secuencia (ST) y 10 complejos clonales (CC), siendo el ST121 y el ST9 los mayoritarios, seguidos del ST87. Se seleccionaron 12 cepas diferentes pertenecientes a estos tres STs, determinándose la presencia de codones de parada prematuros (PMSCs) 6 o 19 en la secuencia de la InlA en el 58,3% de las cepas analizadas. Por el contrario, no se detectaron mutaciones que generasen PMSCs en la secuencia del PrfA. Las cepas ST121 fueron buenas formadoras de biofilm a 22 ºC, aunque ninguna de ellas formó biofilm a 8 ºC. Se determinó la presencia del transposón Tn*6188*, portador de genes de resistencia a desinfectantes como

el cloruro de benzalconio, en el 25,0% de cepas, mientras que no se detectó el casete *bcrABC* en ninguna.

Mediante tratamientos de altas presiones (450 MPa/10 min o 600 MPa/5 min) para inactivar *L. monocytogenes* en jamones deshuesados enteros, se consiguieron reducciones importantes de alrededor de 3,5 unidades logarítmicas con el tratamiento más intenso en el interior de los jamones tras dos meses de almacenamiento a 4 y 12 ºC. Dicho tratamiento fue menos eficaz en la superficie del jamón, lo que se atribuyó a las diferencias fisicoquímicas entre ambas partes del jamón, ya que la mayor concentración de sal en el exterior y, por consiguiente, su menor a_w, podría haber ejercido un efecto baroprotector en *L. monocytogenes*. No obstante, fueron precisamente estas características de la superficie del jamón las que dieron lugar a una disminución en los recuentos del patógeno durante el almacenamiento en muestras control sin tratar e impidieron la recuperación de las células supervivientes en las muestras presurizadas.

Con el fin de analizar en mayor profundidad el posible efecto baroprotector, se modificó la a_w de lonchas de jamón curado sometiéndolas a un equilibrio líquido-gas con una solución salina saturada, hasta alcanzar valores de 0,92, 0,88 y 0,84. Las lonchas se contaminaron con *L. monocytogenes*, se presurizaron (450 MPa/10 min o 600 MPa/5 min), y se conservaron a 4 ºC durante 30 días. Una vez más, el tratamiento más intenso fue el más efectivo, registrándose reducciones de *L. monocytogenes* entre 2,5 y 3,0 unidades logarítmicas un día después de la presurización. Sin embargo, la eficacia del tratamiento disminuyó con el descenso de a_w del jamón, siendo las reducciones con a_w 0,92 más de una unidad logarítmica superiores a las conseguidas con a_w 0,88. Este efecto del tratamiento más intenso en las muestras con mayor a_w se registró también tras 30 días de almacenamiento a 4 ºC, con reducciones entre 3,0 y 3,5 unidades, mientras que la presurización a 450 MPa durante 10 min tuvo una eficacia menor frente al patógeno. En cuanto a los microorganismos totales, se mantuvieron por debajo de 10^6 UFC/g durante los 30 días de ensayo en todas las muestras presurizadas.

Se ensayaron también tratamientos antimicrobianos de bioconservación, de forma individual y en combinación con un tratamiento moderado de altas presiones (450 MPa/10 min), frente a *L. monocytogenes* en jamón curado loncheado que se conservó durante 30 días a 4 y 12 ºC. La presurización y la aplicación individual de timol, así como la combinación de ambos, consiguió reducciones moderadas de *L. monocytogenes*. Además, este aceite esencial afectó al olor del producto. Mediante la aplicación individual de enterocinas A y B se alcanzó una mayor inactivación, con reducciones de 2,5 a 3,0

unidades logarítmicas, pero no se evitó la recuperación del patógeno. Sin embargo, cuando las enterocinas se combinaron con altas presiones, se consiguió un efecto anti-*Listeria* sinérgico, que impidió el crecimiento del patógeno durante 30 días. Este tratamiento combinado mantuvo los microorganismos totales en niveles bajos durante todo el almacenamiento a 4 y 12 ºC, y no causó modificaciones importantes en el pH, la a_w, el color o la textura del jamón curado.

Respecto al efecto de los tratamientos en la expresión génica de *L. monocytogenes*, tanto las altas presiones como las enterocinas A y B produjeron cambios en los patrones de expresión de genes de virulencia y/o resistencia al estrés del patógeno, que fueron específicos de cepa. La aplicación de tratamientos de 450 MPa/10 min o 600 MPa/5 min produjo una represión general de los genes *prfA, plcA, hly, sigB* y *lmo1421* inmediatamente después de su aplicación para una de las cepas ensayadas (serotipo 1/2a), mientras que indujo su expresión para la otra cepa (serotipo 4b), efecto que se atenuó durante el almacenamiento, de forma que, tras 30 días, la expresión de los genes se reprimió, principalmente en las muestras tratadas a 600 MPa durante 5 min. Cuando los tratamientos de alta presión se aplicaron en jamón con distinta a_w (0,92, 0,88 y 0,84), se observó una inducción de la expresión de los genes *plcA, hly, iap* y *sigB* para una cepa de serotipo 1/2b, que, por lo general, fue más pronunciada en muestras con a_w más alta y presurizadas a 450 MPa durante 10 min. Por el contrario, los patrones de expresión de la cepa 1/2c fueron reprimidos durante los 30 días de almacenamiento a 4 ºC, principalmente inmediatamente después de la aplicación de los tratamientos y para muestras con a_w de 0,92 y 0,88. La bioconservación con enterocinas A y B en jamón curado loncheado provocó una sobreexpresión de los genes *prfA, inlA, inlB, clpC* y *fbpA* de una cepa de *L. monocytogenes* de serotipo 1/2a, aunque tras 30 días de almacenamiento a 4 ºC no se apreciaron apenas diferencias entre los patrones de expresión de las muestras tratadas y control. Por otro lado, las enterocinas A y B indujeron la expresión de estos genes para una cepa 4b en las horas siguientes a su aplicación, mientras que a partir del primer día este efecto se atenuó y al final del almacenamiento la mayoría de los genes fueron reprimidos, especialmente a 20 ºC.

L. monocytogenes está presente en los ambientes postprocesado de jamón curado, incluso tras la limpieza y desinfección, por lo que caracterizar las cepas contaminantes permitiría llevar a cabo una estimación del riesgo potencial, así como adoptar distintas estrategias de control. En caso de contaminación del producto, los tratamientos de altas presiones a 600 MPa durante 5 min inactivarían a *L. monocytogenes* en el interior de

jamones deshuesados enteros y en lonchas de jamón, sin comprometer apenas sus características fisicoquímicas, aunque es muy importante tener en cuenta la a_w del alimento, ya que valores bajos ejercen un efecto baroprotector en esta bacteria. Por tanto, la combinación de las altas presiones con enterocinas A y B permitiría no solo solventar este inconveniente, sino también reducir la intensidad de los tratamientos de alta presión, dado su efecto anti-*Listeria* sinérgico. Si bien estas estrategias producen cambios en los patrones de expresión de genes de virulencia y/o resistencia a estrés de *L. monocytogenes*, no se ha encontrado relación con una mayor virulencia potencial de las células supervivientes del patógeno.

Summary

Listeria monocytogenes is a pathogenic bacterium that causes listeriosis, a disease mainly caused by the consumption of contaminated ready-to-eat (RTE) foods, being meat products the most frequently associated with cases of listeriosis. Dry-cured ham is a cured RTE meat product widely accepted in the Spanish market and whose export to other countries has increased significantly in recent years. It is considered a safe product due to its low water activity (a_w) and high salt content, but it can be contaminated with *L. monocytogenes* during the deboning and slicing operations prior to commercialization. European regulations allow up to 100 CFU/g of *L monocytogenes* in products that cannot support its growth or that are not intended for infants and for special medical purposes, while other countries, such as USA, apply more restrictive policies of zero tolerance or absence in 25 g of product. The objectives of this doctoral thesis were: to characterize the presence of *L. monocytogenes* in the deboning and slicing areas of dry-cured ham industries, to apply high-pressure treatments, biopreservation and their combinations to inactivate *L. monocytogenes* in dry-cured ham, and, finally, to investigate whether these treatments affect the expression of stress resistance and/or virulence genes in surviving pathogen cells.

L. monocytogenes was isolated from 11.8% of environmental and surface samples at the deboning areas of three dry-cured ham production plants. Most of the positive samples were taken from non-contact areas with the product, collected during processing, although the pathogen was also isolated after cleaning and disinfection of surfaces and industrial environment. The most common serotype was 1/2a, followed by serotypes 1/2b and 1/2c, while only one isolate ascribed to serotype 4b was detected. Twenty different pulsetypes were identified, four of which were considered persistent. The isolates obtained were grouped into 11 sequence types (ST) and 10 clonal complexes (CC), with ST121 and ST9 being predominant, followed by ST87. Twelve different strains belonging to these three STs were selected, determining the presence of premature stop codons (PMSCs) 6 or 19 in the InlA sequence in 58.3% of the strains analysed. In contrast, no mutations generating PMSCs were detected in the PrfA sequence. ST121 strains were good biofilm formers at 22 °C, although none formed biofilm at 8 °C. The presence of the Tn*6188* transposon, carrying genes for resistance to disinfectants such as benzalkonium chloride, was found in 25.0% of strains, while the *bcrABC* cassette was not detected.

High pressure treatments (450 MPa/10 min or 600 MPa/5 min) to inactivate *L. monocytogenes* in whole deboned hams resulted in significant reductions of about 3.5 log

units at 600 MPa/5 min inside the hams after two months of storage at 4 and 12 °C. This treatment was less effective on the surface of the dry-cured ham, attributed to the physicochemical differences between the two parts of the ham, as the higher salt concentration on the outside and, consequently, lower a_w, might exert a baroprotective effect on *L. monocytogenes*. However, these characteristics of the ham surface led to a decrease in pathogen counts during storage in untreated control samples and prevented the recovery of surviving cells in pressurized samples.

In order to further analyse this baroprotective effect, the a_w of dry-cured ham slices was modified by subjecting them to a liquid-gas equilibrium using a saturated salt solution, until values of 0.92, 0.88 and 0.84 were reached. The slices were contaminated with *L. monocytogenes*, pressurized (450 MPa/10 min or 600 MPa/5 min) and stored at 4°C for 30 days. Again, the most intense treatment was the most effective, with reductions of *L. monocytogenes* between 2.5 and 3.0 log units one day after pressurization. However, the effectiveness of the treatment diminished with decreasing a_w of dry-cured ham, with reductions at a_w 0.92 more than one log unit higher than those achieved at a_w 0.88. This effect was also recorded on samples with higher a_w after 30 days of storage at 4 °C, with reductions between 3.0 and 3.5 units, while pressurization at 450 MPa for 10 min had a lower efficacy against the pathogen. Total viable counts remained below 10^6 CFU/g during the 30 days of the study in all pressurized samples.

Antimicrobial biopreservation treatments, individually and in combination with moderate high-pressure treatment (450 MPa/10 min), were also tested against *L. monocytogenes* in sliced dry-cured ham, which was stored for 30 days at 4 and 12 ºC. Pressurization and individual application of thymol, as well as their combination, achieved moderate reductions of *L. monocytogenes*. In addition, this essential oil affected the odour of the product. Higher inactivation was achieved by individual application of enterocins A and B, with reductions of 2.5 to 3.0 log units, but pathogen recovery was not avoided. However, when enterocins were combined with high pressure, a synergistic anti-*Listeria* effect was attained, which prevented pathogen growth for 30 days. This combined treatment kept total viable counts at low levels throughout storage at 4 and 12°C, without significant changes in pH, a_w, colour or texture of the dry-cured ham.

Regarding the effect of treatments on *L. monocytogenes* gene expression, both high pressure and enterocins A and B produced strain-specific changes in the expression patterns of virulence and/or stress resistance genes of the pathogen. Pressurization at 450 MPa/10 min or 600 MPa/5 min caused a general repression of *prfA*, *plcA*, *hly*, *sigB* and

lmo1421 genes immediately after application for one of the tested strains (serotype 1/2a), while induced their expression for the other strain (serotype 4b), an effect that was attenuated during storage, so that, after 30 days, gene expression was repressed, mainly in the samples treated at 600 MPa for 5 min. When high pressure treatments were applied to ham with different a_w (0.92, 0.88 and 0.84), an induction of *plcA*, *hly*, *iap* and *sigB* gene expression was observed for a serotype 1/2b strain, which was generally more pronounced in samples with higher a_w and pressurized at 450 MPa for 10 min. In contrast, the expression patterns of strain 1/2c were repressed during the 30 days of storage at 4 °C, mainly immediately after application of the treatments and for samples with a_w of 0.92 and 0.88. Biopreservation with enterocins A and B in sliced dry-cured ham resulted in an overexpression of *prfA*, *inlA*, *inlB*, *clpC* and *fbpA* genes of a *L. monocytogenes* serotype 1/2a strain, although after 30 days of storage at 4 °C the differences between the expression patterns of treated and control samples were scarce. On the other hand, enterocins A and B induced the expression of these genes for a strain 4b within hours of application, whereas after the first day this effect was attenuated and at the end of storage most of the genes were repressed, especially at 20 °C.

L. monocytogenes is present in the post-processing environment of dry-cured ham, even after cleaning and disinfection, so characterizing the contaminating strains would allow an estimation of the potential risk and the adoption of different control strategies. In case of product contamination, high pressure treatments at 600 MPa for 5 min would inactivate *L. monocytogenes* inside whole deboned dry-cured hams and ham slices, with little compromise to their physicochemical characteristics. However, dry-cured ham water activity should be considered, as low values exert a baroprotective effect on this bacterium. Therefore, the combination of high pressure with enterocins A and B would allow not only to solve this drawback but also to reduce the intensity of high pressure treatments, as a synergistic anti-*Listeria* effect was demonstrated. Although these strategies produce changes in the expression patterns of virulence genes and/or stress resistance of *L. monocytogenes*, increased virulence potential of pathogen surviving cells was not observed.

Introducción

1. *Listeria monocytogenes*

L. monocytogenes es una bacteria patógena perteneciente al género *Listeria*, un grupo de bacilos Gram-positivos, anaerobios facultativos, no formadores de esporas, sin cápsula, móviles a temperaturas entre 10-25 ºC gracias a la formación de flagelina y con un bajo contenido de G+C (Velge y Roche, 2010). Puede crecer en un amplio intervalo de pH (4,3-9,8) y temperatura (0,5-45 ºC), así como sobrevivir a concentraciones elevadas de sal (hasta 20% peso/volumen) y baja actividad de agua (a_w) (Lado y Yousef, 2007). El género *Listeria* incluye 25 especies, organizadas en dos grupos en base a su relación filogenética, el clado *Listeria sensu stricto*, constituido por las especies *L. monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri, L. marthii, L. farberi, L. immobilis* y *L. cossartiae*, y el clado *Listeria sensu lato*, constituido por *L. grayi*, *L. rocourtiae*, *L. fleischmannii*, *L. weihenstephanensis*, *L. floridensis*, *L. aquatica*, *L. cornellensis*, *L. riparia*, *L. grandensis*, *L. booriae*, *L. newyorkensis*, *L. costaricensis*, *L. goaensis*, *L. thailandensis*, *L portnoyi* y *L. rustica* (Carlin et al., 2021; Leclercq et al., 2019; Orsi y Wiedmann, 2016). Únicamente *L. monocytogenes* y *L. ivanovii* son patógenas, siendo *L. monocytogenes* el principal agente causal en humanos de una enfermedad denominada listeriosis (Liu, 2006).

Las cepas de *L. monocytogenes* pueden diferenciarse en función de las variaciones de sus antígenos somáticos (O) y flagelares (H) detectadas mediante anticuerpos (López et al., 2006). Actualmente, se conocen 15 subtipos del antígeno O (I-XV) y cuatro del antígeno H (a-d), que constituyen los 14 serotipos de *L. monocytogenes* descritos: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e, 4h y 7 (Doumith et al., 2004; Leclercq et al., 2011; Yin et al., 2019). El análisis filogenético de múltiples genes permite agrupar los subtipos de *L. monocytogenes* en cuatro grupos genéticos denominados linajes I, II, III y IV. La mayoría de los aislados pertenecen al linaje I (serotipos 1/2b, 3b, 4b, 4d y 4e) y al linaje II (serotipos 1/2a, 3a, 1/2c y 3c), mientras que los pertenecientes a los linajes III y IV son atípicos y poco frecuentes (Orsi et al., 2011).

1.1. Ciclo patogénico

La entrada de *L. monocytogenes* en el organismo se produce principalmente a través de la barrera intestinal tras la ingestión de alimentos contaminados. *L. monocytogenes* es un patógeno invasivo capaz de inducir su propia internalización en macrófagos y en varios tipos de células que no son normalmente fagocíticas, como células epiteliales, fibroblastos, hepatocitos, células endoteliales, células dendríticas y neuronas (Vázquez-

Boland et al., 2001b). El potencial de *L. monocytogenes* para causar enfermedad depende de su capacidad para sobrevivir, invadir y replicarse en estas células, y para cruzar la barrera intestinal, el torrente sanguíneo y la barrera placentaria (Cossart, 2011). El ciclo comienza con la adhesión de la bacteria a la superficie de la célula eucariota y la subsiguiente penetración en la célula hospedadora. La entrada en células no fagocíticas se da por un mecanismo de tipo "cierre" y es mediada por dos proteínas de superficie, las internalinas A y B (InlA e InlB), que interaccionan con receptores específicos de la célula hospedadora (Figura 1, 1). La acción de los factores listeriolisina O (LLO) y las fosfolipasas A y B (PlcA y PlcB), esta última tras activación previa por una metaloproteasa (Mpl), daña la membrana de la vacuola fagocítica, provocando su lisis (Figura 1, 2). Una vez en el citosol, *L. monocytogenes* expresa los genes necesarios para adquirir nutrientes y empezar una rápida multiplicación celular, entre ellos *hpt*, codificante de la permeasa Hpt transportadora de hexosa-6-fosfato, importante para el crecimiento celular (Figura 1, 3). Posteriormente, se induce la formación de filamentos de actina en un polo de la bacteria gracias a la proteína ActA, lo que permite el movimiento intracelular hacia las células vecinas y la diseminación de *L. monocytogenes* célula a célula, que se rodea a su paso de una doble membrana (Figura 1, 4-7) (Rolhion y Cossart, 2017). Esta doble membrana se rompe finalmente por la acción de LLO, PlcA y PlcB, y se inicia un nuevo ciclo de infección (Figura 1, 8).

1.2. Genes de invasión, virulencia y resistencia al estrés

La adhesión de *L. monocytogenes* a la célula hospedadora es una etapa fundamental para la patogenicidad de la bacteria. Se han descrito varios factores que permiten esta adhesión, entre los que se encuentran proteínas como FbpA y ClpC. FbpA es una proteína de superficie homóloga a las proteínas de unión a fibronectina, que parece actuar como chaperona promoviendo la estabilización o secreción correcta de algunos factores de virulencia como InlB y LLO (Dramsi et al., 2004), mientras que ClpC es una ATPasa involucrada en la adhesión e invasión celular, cuya ausencia provoca una reducción de la transcripción de otras proteínas relacionadas con la virulencia del patógeno, como las internalinas A y B y la ActA (Nair et al., 2000). Además, este mediador participa también en el escape del fagosoma y en la multiplicación intracelular (Vera et al., 2013). Tras la adhesión, la InlA se une al receptor E-cadherina, que es una glicoproteína transmembrana presente en la superficie de varios tipos de células, incluidos enterocitos.

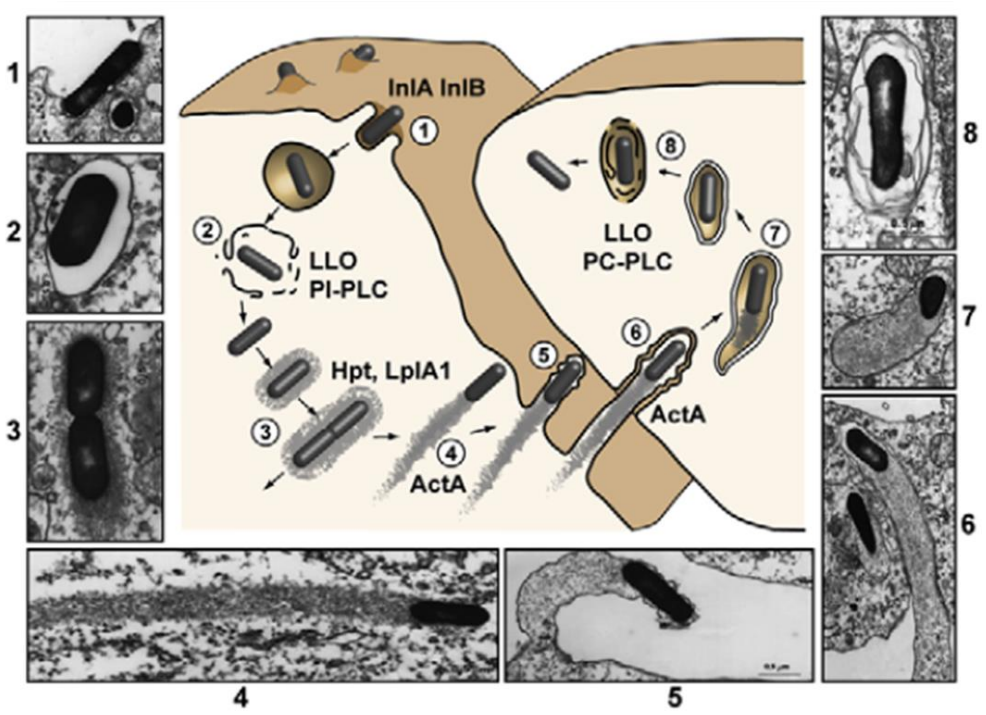

Figura 1. Representación esquemática de los pasos del ciclo celular infectivo de *L. monocytogenes* en el que se muestran los principales factores de virulencia. Cada paso del ciclo se ilustra con imágenes de microscopía electrónica (Cossart y Toledo-Arana, 2008).

Asimismo, la InlB es capaz de unirse a varios receptores, entre ellos el receptor del factor de crecimiento del hepatocito, Met, que se expresa ubicuamente permitiendo que *L. monocytogenes* se internalice en gran variedad de células. Las modificaciones postraduccionales que sufren los receptores de estas dos proteínas son fundamentales para el proceso de internalización, lo que hace que las internalinas A y B sean los principales factores de virulencia implicados en la invasión celular, sobre todo en células no fagocíticas (Pizarro-Cerdá et al., 2012). Otro gen relevante relacionado inicialmente con la invasión celular es el *iap*, que codifica una proteína extracelular denominada p60. Actualmente se sabe que actúa como una hidrolasa y que está implicada en los últimos pasos de la división celular (Pilgrim et al., 2003; Vera et al., 2013).

Seis de los factores de virulencia responsables de la vida parasitaria de *L. monocytogenes* (PrfA, PlcA, LLO, Mpl, ActA y PlcB) están codificados por genes que se encuentran situados en una región de 9 kb del cromosoma denominada isla de patogenicidad 1 (LIPI-1). PrfA es considerado el principal regulador de la virulencia de *L. monocytogenes*, ya que regula la activación transcripcional de todos los genes del

clúster, incluida la suya propia, así como la de otros genes situados en otros lugares del cromosoma, como es el caso de las internalinas A, B, C y J, y la permeasa Hpt (Vázquez-Boland et al., 2001b). Esta expresión dependiente de PrfA se determina primariamente por la concentración de PrfA y su afinidad por los promotores de los genes que activa. El factor PrfA puede regular negativamente su propia síntesis cuando la bacteria se encuentra en su vida saprofítica. También se ha visto una regulación indirecta de PrfA en genes que codifican transportadores, enzimas metabólicas, reguladores y otras proteínas de función desconocida, lo que indica que podría ejercer efectos generales en la homeostasis del patógeno (De las Heras et al., 2011). La acción de este gen, junto con la de *hly* y *actA*, se considera fundamental en la patogenicidad de *L. monocytogenes*. El gen *hly* codifica la hemolisina formadora de poros LLO, necesaria para la lisis de la vacuola y la liberación de la bacteria, mientras que el gen *actA* codifica la proteína de superficie ActA, que promueve los movimientos citoplasmáticos y la diseminación de la bacteria (Vázquez-Boland et al., 2001a, b). La función de las fosfolipasas A y B, junto con LLO, consiste en disolver la membrana de la vacuola primaria formada tras la fagocitosis de la bacteria extracelular, aunque la principal función de la fosfolipasa B es disgregar la doble membrana del fagosoma secundario formado tras la diseminación célula a célula (Vázquez-Boland et al., 2001a, b).

La expresión de genes de resistencia a estrés, que a su vez regulan la expresión coordinada de algunos genes de virulencia, promueve la adaptación de la bacteria al paso de la vida saprofítica a la parasitaria, así como a la gran variedad de condiciones que se dan durante la infección y la transmisión del patógeno de un hospedador a otro (Domínguez-Bernal, 2001). El factor alternativo SigB, σ^B, codificado por el gen *sigB*, es un regulador de estrés que contribuye a la supervivencia de *L. monocytogenes* ante distintas condiciones ambientales como estrés osmótico (Sue et al., 2003), ácido (Wemekamp-Kamphuis et al., 2004), exposición a bilis (Zhang et al., 2011) o a antimicrobianos que dañan la pared celular (Begley et al., 2006). Además, juega un papel muy importante en la virulencia, ya que regula la transcripción de PrfA asegurándose de mantener niveles basales y flexibles de la proteína bajo gran variedad de situaciones (De las Heras et al., 2011), y de otros genes de virulencia como *inlA* e *inlB* (Kazmierczak et al., 2006). SigB también regula la expresión de otros genes de resistencia a estrés, como es el caso de *lmo1421*, que codifica un transportador ABC (*ATP binding cassette* o dominio de unión a ATP) de glicina/betaína relacionado con respuesta general a estrés (Sue et al., 2003).

En algunas cepas de *L. monocytogenes*, pertenecientes al linaje I y relacionadas con brotes de listeriosis, se ha observado una isla de patogenicidad 3 (LIPI-3) que contiene el gen codificante de una segunda hemolisina, denominada listeriolisina S o LLS, responsable del aumento de la virulencia en dichas cepas (Cotter et al., 2008). Asimismo, se ha propuesto considerar una cuarta isla de patogenicidad de *L. monocytogenes* (LIPI-4), con un clúster de genes implicado en daños del sistema nervioso central y la unidad feto-placentaria (Maury et al., 2016).

1.3. Listeriosis

El consumo de alimentos contaminados con *L. monocytogenes* puede causar listeriosis. La gravedad de la enfermedad depende del estado inmunológico del hospedador, el potencial patogénico de la cepa y la dosis infecciosa. Así, en individuos sanos la listeriosis suele cursar de forma leve no invasiva, como una gastroenteritis febril, con un período de incubación de 1 a 7 días (Warriner y Namvar, 2009). En los grupos de riesgo como niños, ancianos, mujeres embarazadas e individuos inmunodeprimidos, esta enfermedad suele cursar de forma grave como una listeriosis invasiva, una de las infecciones alimentarias más severas en la Unión Europea (UE) en términos de hospitalización, con más del 92% de los casos notificados en 2019 (EFSA y ECDC, 2021). Este tipo de listeriosis, con un período de incubación más largo que varía generalmente entre 1 y 3 semanas, se suele relacionar con septicemia, meningitis y encefalitis, y abortos, nacidos muertos o nacimientos prematuros en caso de mujeres embarazadas. La listeriosis invasiva tiene una baja morbilidad pero presenta la mayor tasa de mortalidad de las infecciones transmitidas por alimentos en países industrializados, que en 2019 fue del 17,6% en la UE. En la última década, se ha observado una tendencia creciente en los casos de listeriosis en la UE (Figura 2). Concretamente, en 2019 se notificaron 2 621 casos, siendo las personas de edad avanzada las más afectadas, con una tasa de mortalidad superior al 19% en los mayores de 64 años y superior al 23% en los mayores de 84 años (EFSA y ECDC, 2021).

El 99% de casos de listeriosis se atribuye al consumo de alimentos contaminados (Scallan et al., 2011), siendo los listos para el consumo o RTE los implicados con mayor frecuencia en brotes de la enfermedad (EFSA y ECDC, 2021). Los alimentos RTE son definidos por el Reglamento (CE) nº 2073/2005 como alimentos destinados por el productor o el fabricante al consumo humano directo sin necesidad de cocinado u otro tipo de transformación eficaz para eliminar o reducir a un nivel aceptable los

microorganismos peligrosos (EC, 2005). La mayor incidencia de *L. monocytogenes* en alimentos RTE analizados en la UE durante 2019 se determinó en productos pesqueros (4,3%), seguida de productos cárnicos (2,9%) y de leche y productos lácteos (< 1%). El 51,4% de los productos cárnicos RTE en los que se detectó *L. monocytogenes* fue de origen porcino (EFSA y ECDC, 2021).

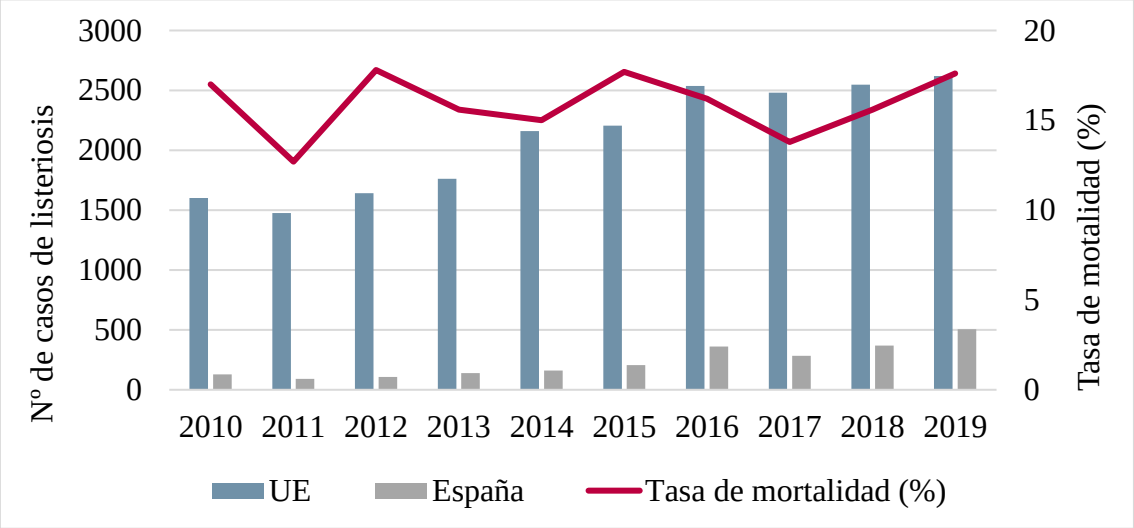

Figura 2. Número de casos confirmados de listeriosis en la UE y España y tasa de mortalidad en la UE durante el período 2010-2019 (EFSA y ECDC, 2012, 2013, 2014, 2015a, 2015b, 2016, 2017, 2018, 2019, 2021).

Durante ese mismo año, y al igual que en años anteriores, la combinación *L. monocytogenes*/productos cárnicos fue la responsable de la mayoría de muertes por consumo de alimentos y agua contaminados (EFSA y ECDC, 2021) (Tabla 1). Algunos productos cárnicos relacionados con brotes de listeriosis han sido carne de pavo en EE. UU. en 2005 (Olsen et al., 2005), productos cárnicos RTE en Nueva Zelanda en 2012 (Rivas et al., 2019) y en Sudáfrica en 2017 (Thomas et al., 2020), o carne mechada en España en 2019, el mayor brote registrado en nuestro país hasta la fecha y uno de los mayores de Europa, con 225 casos, 131 hospitalizaciones, 3 muertes y 6 abortos (EFSA y ECDC, 2021).

2. *L. monocytogenes* en alimentos

L. monocytogenes puede sobrevivir y crecer durante la producción, conservación y almacenamiento de los alimentos. Por su ubicuidad, su carácter psicrotrofo, su resistencia a agentes desinfectantes, su capacidad de formar biofilms y su tolerancia a la sal y nitritos,

Tabla 1. Combinaciones patógeno/vehículo alimentario causantes del mayor número de muertes en la Unión Europea durante 2019 (EFSA y ECDC, 2021).

Clasificación	Agente causal	Alimento	Muertes
1	*L. monocytogenes*	Carne y productos cárnicos	20[a]
2	*Clostridium perfringens*	Alimentos de origen no animal	2
3	Toxinas bacterianas	Otros alimentos	1
3	*Salmonella* spp.	Huevos y ovoproductos	1
3	*Bacillus cereus*	Otros alimentos	1
3	*Escherichia coli* productora de toxina Shiga	Leche y productos lácteos	1
3	*Clostridium botulinum*	Otros alimentos	1
3	*Clostridium perfringens*	Carne y productos cárnicos	1
3	*Salmonella* spp.	Carne y productos cárnicos	1

[a]Las muertes registradas fueron: nueve en Reino Unido, seis en Países Bajos, tres en España y dos en Italia. El número de muertes por *L. monocytogenes* asociada a carne y productos cárnicos se incrementó en 2019 con respecto al período 2010-2018, en el que el valor medio de muertes por año fue 1,8.

L. monocytogenes es seguramente una de las bacterias patógenas que más preocupan a la industria alimentaria, que demanda soluciones para ofrecer productos seguros y de calidad, sin que resulten afectadas sus características sensoriales. Los alimentos RTE, poco procesados y con larga vida útil a temperaturas de refrigeración, son susceptibles de contaminación por *L. monocytogenes* durante su elaboración, especialmente en las etapas de postprocesado (Hereu et al., 2012). Algunos alimentos RTE como los lácteos, productos cárnicos, pescado ahumado y marisco, así como alimentos de origen vegetal como setas, melones o manzanas caramelizadas han sido asociados con casos y/o brotes por *L. monocytogenes* (Farber et al., 2020). Por tanto, es importante identificar los vehículos de transmisión del patógeno y evitar la contaminación del producto desde el inicio de su producción en las industrias alimentarias.

2.1. Fuentes de contaminación

L. monocytogenes se encuentra habitualmente en el suelo, la vegetación en descomposición, las aguas residuales, el agua dulce y en heces humanas y animales, en diferentes entornos naturales, urbanos, agrícolas y ambientes de procesado de alimentos (Ferreira et al., 2014). Puede introducirse en las plantas de procesado de alimentos a través de las materias primas, la maquinaria y los operarios (Ricci et al., 2018), y puede colonizar el ambiente y los equipos, persistiendo en las instalaciones durante períodos prolongados de tiempo, contaminando los productos finales durante las últimas etapas del procesado (Sereno et al., 2019). Cualquier cepa de *L. monocytogenes* podría llegar a ser persistente si se diesen las condiciones oportunas (Carpentier y Cerf, 2011; Ferreira et al., 2011). La persistencia se relaciona en gran medida con la existencia de zonas de difícil limpieza, así como con la capacidad de las cepas de formar biofilms y/o de resistir a los desinfectantes (Lee et al., 2019; Martínez-Suárez et al., 2016; Stoller et al., 2019). Así, cepas persistentes y ampliamente distribuidas en el ambiente de las industrias alimentarias han mostrado una mayor capacidad de formación de biofilms (Borucki et al., 2003). Las células de *L. monocytogenes* son capaces de agruparse y adherirse a superficies de materiales utilizados en la industria, lo que les proporciona una serie de ventajas ecológicas, como una mayor tolerancia a desinfectantes, que favorece su persistencia (Bridier et al., 2011; Colagiorgi et al., 2017). El aumento de la tolerancia a desinfectantes podría deberse a la limitación de la difusión de agentes antimicrobianos por parte de la matriz extracelular del biofilm, que actuaría como barrera, a una adaptación fenotípica que sufrirían las células en ese estado y que se podría perder si las células volviesen a su estado planctónico, o incluso a la transferencia de genes de resistencia a desinfectantes entre las células (Bridier et al., 2011; Ruiz-Bolivar et al., 2008). En general, la tolerancia de cepas de *L. monocytogenes* a desinfectantes utilizados en la industria puede deberse a varios factores, entre ellos la presencia repetida y frecuente de concentraciones subinhibitorias de estos productos que pueden dar lugar a cambios genéticos en las cepas que a su vez pueden generar una reducción de la permeabilidad de las membranas del patógeno a estos compuestos, así como a la presencia de genes de resistencia a desinfectantes, cromosómicos o adquiridos mediante plásmidos, la transición a un estado viable no cultivable o la formación de biofilms (Figura 3) (Duze et al., 2021; Elhanafi et al., 2010; Martínez-Suárez et al., 2016; Müller et al., 2013; Noll et al., 2020).

En cualquier caso, la persistencia de las cepas en las industrias parece ser resultado de la combinación de factores genéticos y ambientales. Se han encontrado cepas de *L.*

monocytogenes que permanecen durante años en industrias productoras de alimentos cárnicos RTE (Ferreira et al., 2011; Ortiz et al., 2010) e incluso se ha detectado la presencia de la misma cepa en distintas plantas de procesado de alimentos dispersas geográficamente (Chasseignaux et al., 2001; D'Arrigo et al., 2020; Leong et al., 2014; Morganti et al., 2016). Por tanto, disponer de técnicas eficaces que permitan la detección y caracterización del patógeno en los equipos e instalaciones de las industrias resulta crucial para poder implementar estrategias de control que impidan que el patógeno alcance el producto final.

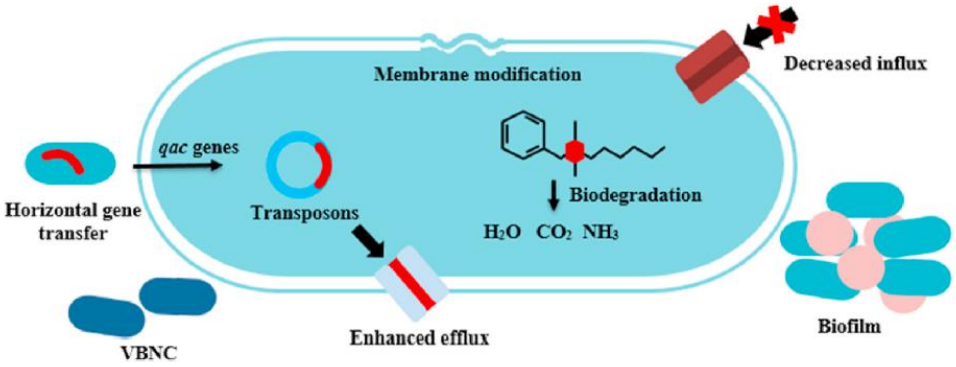

Figura 3. Mecanismos de tolerancia a desinfectantes de *L. monocytogenes*, entre los que se incluyen la transición al estado viable pero no cultivable (VBNC), la formación de biofilms, la modificación de la superficie celular y la activación de bombas de eflujo cromosómicas o adquiridas mediante elementos genéticos móviles (Duze et al., 2021).

2.2. Detección y caracterización

El Reglamento (CE) nº 852/2004, relativo a la higiene de los productos alimenticios, obliga a la implantación de sistemas de APPCC (Análisis de Peligros y Puntos de Control Crítico) por parte de las industrias alimentarias, mientras que el Reglamento (CE) nº 2073/2005 obliga a la toma de muestras ambientales y de equipos de procesado de alimentos para detectar *L. monocytogenes* (EC, 2004, 2005). Es, por tanto, fundamental disponer de métodos rápidos y sensibles para la detección, identificación y caracterización de *L. monocytogenes* en alimentos (materias primas y productos intermedios y finales) y ambiente de las plantas de procesado. Entre estos métodos se encuentran las normas ISO 11290-1 y 11290-2 (ISO, 2017a, b), el desarrollado por la FDA de EE. UU. (Hitchins et al., 2017), o el método del Departamento de Agricultura de EE. UU. (USDA-FSIS, 2021). El método del USDA-FSIS es el utilizado habitualmente

para el aislamiento y la identificación de *L. monocytogenes* en productos cárnicos, bien sea crudos o RTE, y consiste en someter a la muestra a un enriquecimiento primario o preenriquecimiento en caldo UVM, seguido de un enriquecimiento secundario en medio líquido MOPS-BLEB. Posteriormente, se aísla la bacteria en medio sólido selectivo MOX. Tras el aislamiento, es necesaria una confirmación e identificación de los aislados, que puede hacerse de manera convencional mediante cultivo microbiológico y análisis bioquímico o fenotípico, o mediante métodos rápidos válidos tanto para detección como para confirmación/identificación, como es el caso de las técnicas inmunológicas o moleculares. Entre estas últimas, la PCR o qPCR (PCR cuantitativa), permiten la identificación de *L. monocytogenes* directamente del alimento, siendo la segunda más directa y específica a la hora de cuantificar, permitiendo incluso la búsqueda y cuantificación conjunta de diversos patógenos (Cocolin et al., 2002, 2011; Rantsiou et al., 2008; Rodríguez-Lázaro et al., 2004; Wei et al., 2018).

Tras la identificación, se lleva a cabo una caracterización de *L. monocytogenes*, que permite trazar las rutas de contaminación de los productos. Una de las técnicas más utilizadas es el análisis de serotipos mediante PCR multiplex (Doumith et al., 2004), que permite diferenciar los serogrupos IIa, IIb, IIc y IVb, que presentan la mayoría de los aislados alimentarios y clínicos del patógeno. La electroforesis en gel de campo pulsado o PFGE se considera el test de referencia para la tipificación de *L. monocytogenes* por ser una técnica altamente discriminatoria (CDC, 2017). El método estandarizado del CDC de EE. UU. se basa en la lisis celular y digestión del ADN con dos enzimas de restricción (AscI y ApaI) con secuencias de reconocimiento poco frecuentes que generan fragmentos de gran tamaño, los cuales son separados mediante electroforesis en gel de agarosa utilizando un gradiente de pulsos eléctricos (Halpin et al., 2010). La relación filogenética entre aislados se evalúa en función de la similitud de los patrones de bandas resultantes. El CDC creó la red internacional PulseNet (https://www.pulsenetinternational.org), en la que participan laboratorios del sistema sanitario público y agencias alimentarias, con el fin de identificar subtipos y detectar y realizar el seguimiento de brotes de infecciones de origen alimentario. En la actualidad se utilizan también otras técnicas basadas en secuenciación, como la tipificación de secuencias multilocus (MLST) o la tipificación de secuencias de virulencia multilocus (MVLST), que detectan polimorfismos de un solo nucleótido o SNP en un fragmento de pequeño tamaño de varios genes constitutivos esenciales de la célula que están muy conservados o en genes de virulencia, respectivamente. Uno de los protocolos de MLST más utilizados para tipificación de *L.*

monocytogenes consiste en el análisis de fragmentos de siete genes: *abcZ*, codificante de un transportador ABC y *bglA*, *cat*, *dapE*, *dat*, *ldh* y *lhkA*, codificantes de las enzimas β-glucosidasa, catalasa, succinil diaminopimelato desuccinilasa, aminotransferasa de D-aminoácidos, lactato deshidrogenasa e histidina quinasa, respectivamente (Ragon et al., 2008). Existe una base de datos creada por el Instituto Pasteur que permite asignar los tipos de secuencia o ST analizados y comparar los perfiles de MLST de distintos laboratorios (https://www.pasteur.fr/mlst). Otras opciones de tipificación son la secuenciación del genoma completo (WGS), cada vez más utilizada para estudios epidemiológicos y detección de brotes (Farber et al., 2020) o el análisis del número variable de repeticiones en tándem (MLVA) (Martín et al., 2018).

2.3. Variabilidad de la virulencia

Los serotipos de *L. monocytogenes* 1/2a, 1/2b y 4b causan más del 95% de los casos de listeriosis (Kathariou, 2002). Por lo general, las cepas del linaje I se relacionan más con casos y aislados clínicos, mientras que las del linaje II son más características de alimentos y plantas de procesado. Existe una gran variabilidad en la virulencia y patogenicidad de las distintas cepas de *L. monocytogenes*. Concretamente, la atenuación de la virulencia en cepas del linaje II se ha relacionado con mutaciones puntuales en los genes *inlA* y *prfA*, que darían lugar a proteínas truncadas o no funcionales (Roche et al., 2012). En este sentido, se habla de cepas hiper e hipovirulentas, siendo el grupo de virulencia atenuada más relevante aquel en el que se han descrito codones de parada prematuros (PMSCs) en el gen *inlA* (Farber et al., 2020). Se conocen al menos 21 polimorfismos en el gen *inlA* que provocan PMSCs que generan proteínas InlA truncadas (Gelbíčová et al., 2015, 2016; Van Stelten et al., 2010), principalmente en cepas 1/2a y 1/2c aisladas de alimentos. Chen et al. (2011) concluyeron que cepas de *L. monocytogenes* con una InlA truncada podrían ser hasta 10 000 veces menos virulentas que las que presentan la proteína completa. Estas mutaciones reducen significativamente la capacidad de *L. monocytogenes* de invadir células epiteliales intestinales humanas Caco-2 (Ferreira da Silva et al., 2017; Nightingale et al., 2005; Olier et al., 2002, 2003) y parecen ser responsables de la atenuación de la virulencia en un modelo de conejo de indias (Roldgaard et al., 2009).

Distintas mutaciones en el gen *prfA* también se han relacionado ampliamente con una atenuación de la virulencia. Se han descrito polimorfismos que dan lugar a PMSCs o que afectan a dominios necesarios para la actividad de PrfA, lo que genera proteínas

truncadas (Velge et al., 2007). Algunos estudios afirman que las cepas que carecen de *prfA* son prácticamente avirulentas en modelos murinos (Chakraborty et al., 1992; Mengaud et al., 1991; Xayarath y Freitag, 2012). No obstante, estas mutaciones en el *prfA* son menos frecuentes y, por tanto, menos relevantes desde el punto de vista de la salud pública (Farber et al., 2020).

3. *L. monocytogenes* en jamón curado

La industria cárnica representa el cuarto sector industrial más importante de España y el primero dentro de la industria española de alimentos y bebidas (AECOC, 2018). España es el cuarto productor mundial de carne de porcino y destaca por productos de alto valor como paletas y jamones curados (ANICE, 2021). El jamón curado es un producto emblemático entre los productos cárnicos españoles y su producción comprende el 20% de las compañías del sector cárnico español. Dicha producción ha aumentado un 25% entre 2010 y 2018, con una mayor elaboración de jamón curado de cerdo blanco (Mercasa, 2019). Su exportación, que también se ha incrementado en unas 30 000 toneladas en esos mismos años, se produce sobre todo a países europeos, entre los que destacan Francia y Alemania, y otros como Reino Unido, México, EE. UU., Chile, Brasil, Cuba, República Dominicana, China, Japón y Australia (Mercasa, 2019; Rodríguez, 2018).

El jamón curado es un producto elaborado con la extremidad posterior del cerdo que ha sido sometida, con carácter general, a un proceso que comprende varias etapas, el salado, el lavado, el reposo o postsalado y el secado y maduración durante el tiempo suficiente para conferirle las características organolépticas propias (RD 474/2014, 2014). Durante la fase de salado la pieza se cubre de sal, con o sin presencia de sales nitrificantes, y permanece a temperaturas de refrigeración de entre 1 y 3 ºC y una humedad relativa mayor o igual al 75% durante un período de tiempo determinado, generalmente un día por kg de peso. La difusión de sal hacia el interior hace que disminuya la a_w y promueve la deshidratación del pernil, que pierde durante esta etapa del 3 al 4% de su peso. De esta manera se estabiliza microbiológica y enzimáticamente al tiempo que se produce el sabor típico salado. Posteriormente, el jamón se lava para eliminar la sal del exterior. Hasta este momento, las sales se han concentrado sobre todo en la parte superficial, por lo que se requiere una etapa de postsalado para que la sal difunda de manera homogénea hacia el interior de la pieza. Es un proceso lento que dura entre 40 y 90 días y en que la humedad relativa se mantiene en torno al 90-95% y la temperatura entre 1 y 5 ºC hasta que la a_w

alcanza valores inferiores a 0,96 y el pernil pierde del 4 al 6% de su peso. En este punto, la bajada de a_w, la difusión de la sal y la presencia de sales nitrificantes disminuyen el riesgo asociado a la presencia de microorganismos patógenos y alterantes, lo que permite subir la temperatura en las etapas posteriores. En la fase de secado-maduración, la temperatura se incrementa de forma lenta y progresiva hasta alcanzar valores máximos de entre 28 y 34 ºC. La humedad relativa se mantiene entre el 60 y el 80%, continuando así la deshidratación y se alcanza aproximadamente un 32% de pérdida de peso, por lo que la sal se concentra en el interior y disminuye aún más la a_w. En esta etapa tienen lugar las reacciones necesarias (proteolisis, lipolisis, oxidación lipídica, reacciones de Maillard y degradación de Strecker) que generan el color y los olores y sabores típicos del producto. En ocasiones el jamón se mantiene en bodega para obtener una mayor calidad y un sabor más intenso (Arnau, 2014; Santos, 2012; Toldrá, 2007; Toldrá y Aristoy, 2010; Toldrá y Flores, 1998).

El jamón curado es un producto cárnico RTE que por sus características fisicoquímicas se considera seguro desde el punto de vista microbiológico (Reynolds et al., 2001). Sin embargo, durante las operaciones postprocesado, como deshuesado y loncheado, el producto puede contaminarse con microorganismos patógenos del ambiente industrial, como *L. monocytogenes* (Hereu et al., 2012). Se han descrito contaminaciones de *L. monocytogenes* en diferentes zonas de industrias elaboradoras de jamón curado y otros productos cárnicos, incluyendo las zonas de loncheado (Zhu et al., 2005), así como una mayor incidencia en las zonas de deshuesado (Morganti et al., 2016). En 2018, el jamón deshuesado supuso el 77% de las toneladas exportadas de jamón (Mercasa, 2019; Rodríguez, 2018). Por su parte, el 45,5% de los consumidores españoles prefieren el jamón curado loncheado, además de ser el formato que permite al empresario obtener un mayor beneficio económico (Rodríguez, 2018).

Los valores de a_w del jamón curado loncheado distribuido comercialmente son muy variables, presentando en numerosas ocasiones cifras superiores a 0,92. Además, la a_w puede variar entre lonchas e incluso dentro de una misma loncha entre los distintos músculos. En un trabajo realizado por Hereu (2014) se analizó este parámetro en 62 muestras comerciales de jamón curado loncheado y concluyeron que el 10% presentaba valores de a_w mayores o iguales a 0,94, el 80% entre 0,93 y 0,90 y el 10% entre 0,89 y 0,86. Por otra parte, la industria actualmente tiende a reducir el contenido en cloruro sódico, lo que implicaría en el jamón curado un aumento de la a_w, así como a limitar o eliminar el uso de agentes nitrificantes, compuestos que se caracterizan por sus

propiedades antimicrobianas. Todo ello, sumado a un almacenamiento incorrecto en condiciones de abuso de temperatura, podría comprometer la seguridad microbiológica del producto, favoreciendo el crecimiento de *L. monocytogenes*.

Los criterios de seguridad microbiológica respecto a *L. monocytogenes* son variables dependiendo del país. Así, en países pertenecientes a la UE y para los productos cárnicos curados, el Reglamento (CE) nº 1441/2007 establece un máximo de 100 UFC/g durante la vida útil de los productos no destinados a la población de riesgo (EC, 2007). Sin embargo, en otros países con políticas más restrictivas como EE. UU., el criterio es de tolerancia cero o ausencia en 25 g (USDA-FSIS, 2014), lo que podría suponer una barrera a la creciente exportación de productos cárnicos curados tradicionales españoles.

Estas circunstancias hacen necesario disponer de métodos de inactivación de *L. monocytogenes* en jamón curado en caso de que el patógeno alcance el producto final, que sean efectivos y no modifiquen sus características ni propiedades organolépticas.

4. Estrategias de control de *L. monocytogenes*

Los métodos tradicionales de conservación de alimentos basados en tratamientos térmicos resultan muy eficaces en el control de microorganismos patógenos, pero presentan una serie de desventajas. Los tratamientos a altas temperaturas confieren estabilidad y una larga vida útil a los alimentos, pero conllevan la pérdida de nutrientes sensibles al calor, desnaturalización de proteínas, cambios en color, textura, sabor e incluso formación de nuevos compuestos. Los tratamientos menos intensos de pasteurización minimizan las desventajas de los tratamientos a temperaturas más elevadas pero limitan la vida útil de los productos incluso en condiciones de refrigeración (Ray, 2005). Esto, junto a la demanda creciente por parte de los consumidores de alimentos mínimamente procesados con una larga vida útil y sin aditivos, ha potenciado el desarrollo de tratamientos no térmicos que permitan controlar el crecimiento de microorganismos patógenos sin alterar las características sensoriales de los productos.

4.1. Altas presiones hidrostáticas

El tratamiento por altas presiones hidrostáticas (APH) es un proceso físico no térmico que consiste en someter al alimento, envasado en un material flexible, a altos niveles de presión isostática cuyo fluido transmisor suele ser el agua (presión hidrostática) durante unos segundos o minutos. Dicha presión se transmite de forma uniforme e instantánea a todos los puntos del producto, consiguiendo así un efecto equivalente a una

pasteurización, pero sin utilizar calor. Permite la inactivación de microorganismos patógenos y alterantes en alimentos, ampliando su vida útil y mejorando su seguridad microbiológica, sin afectar apenas a sus propiedades organolépticas y nutricionales.

Actualmente, las APH se aplican en gran variedad de alimentos como cárnicos, mermeladas y zumos de frutas, aderezos para ensalada, lácteos, pescados y mariscos, productos con base de aguacate, y más recientemente en salsas vegetales, platos preparados, productos infantiles o alimentación de mascotas (Hiperbaric, 2021). Aproximadamente el 30% de equipos de alta presión (Figura 4) se utilizan en la industria cárnica para conseguir productos con mayor vida útil (Sazonova et al., 2017), entre los que se encuentran jamón cocido, jamón curado loncheado, productos cárnicos fermentados, cortes de pollo o pavo, salchichas, platos RTE, etc. La intensidad de los tratamientos oscila entre 400 y 600 MPa con tiempos cortos de presurización de entre 3 y 7 min a temperatura ambiente, condiciones que conllevan una reducción de microorganismos patógenos y alterantes. No obstante, la eficacia de las APH depende de tres parámetros críticos en el diseño del tratamiento, la presión, el tiempo y la temperatura (Ray, 2005) así como del tipo de microorganismo y su fase de crecimiento y, en gran medida, de la composición y las características fisicoquímicas de la matriz alimentaria.

Figura 4. Equipo de altas presiones comercializado por la empresa Hiperbaric.

En general, las bacterias Gram-positivas son más resistentes a las APH que las Gram-negativas, los cocos más resistentes que los bacilos y las bacterias en fase estacionaria más resistentes que en fase exponencial (Huang et al., 2014). La principal

hipótesis del efecto de las APH en los microorganismos reside en el daño generado en la membrana plasmática, que perdería su fluidez, dando lugar a su ruptura y a la desnaturalización de proteínas de membrana, y su permeabilidad, provocando fugas de material citoplasmático y la destrucción celular (San Martín et al., 2002). En concreto, frente a *L. monocyogenes*, las APH provocan cambios morfológicos, estructurales y fisiológicos, así como alteraciones y daño genético (Figura 5) (Ritz et al., 2002; Tholozan et al., 2000; Van Boeijen et al., 2013).

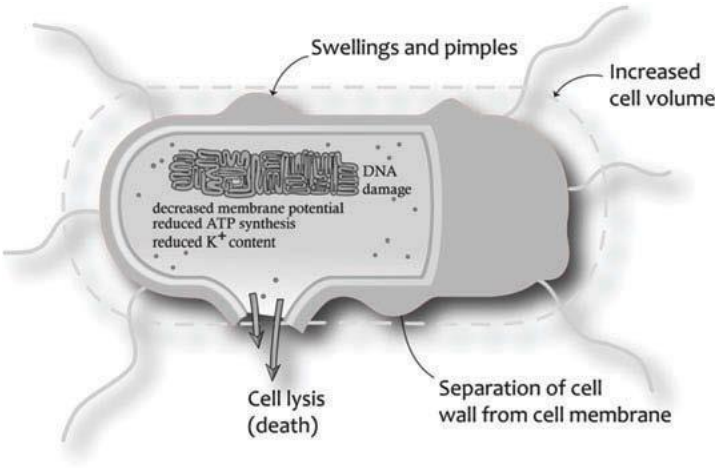

Figura 5. Efecto de las altas presiones en el género *Listeria* (Ferreira et al., 2016).

La sensibilidad de los microorganismos a las APH depende en gran medida de las características del alimento. Un pH ácido puede aumentar el efecto inhibitorio de las altas presiones (Alpas et al., 2000), mientras que una baja a_w ejerce un efecto baroprotector en los microorganismos (Campus, 2010; Patterson, 2005). En el caso del jamón curado, se ha comprobado que la grasa y la baja a_w disminuyen la eficacia de las APH sobre *L. monocytogenes* (Bover-Cid et al., 2015).

Las APH pueden afectar a las características del jamón curado. Así, vitaminas, pigmentos y otras moléculas de bajo peso molecular responsables del sabor y del aroma no resultan afectadas, ya que las APH no actúan sobre enlaces covalentes (Ray, 2005). Sin embargo, pueden causar desnaturalización total o parcial de proteínas cárnicas (De Alba et al., 2015), incluyendo la degradación de la actina en jamón curado (López-Pedrouso et al., 2019), que podría suponer un cambio en las propiedades gelificantes (Jiménez, 2002) y provocar modificaciones en la textura del jamón. El color característico puede resultar también afectado por el tratamiento. En este sentido, se ha observado un ligero aumento de la dureza y la luminosidad (L^*), así como una disminución de la

terneza, la jugosidad y el color rojo (*a**) en jamón curado presurizado (Clariana et al., 2011; Coll-Brasas et al., 2019; Fuentes et al., 2010). No obstante, los cambios producidos en el color y la textura como consecuencia del tratamiento son, en general, aceptables en este tipo de productos (Ferrini et al., 2012) y es cada vez más frecuente la comercialización de jamón curado loncheado sometido a altas presiones.

4.2. Bioconservación

La bioconservación consiste en una forma biotecnológica de conservación que explota la capacidad de microorganismos reconocidos como seguros, GRAS (*Generally Recognized As Safe*) por la FDA o QPS (*Qualified Presumption of Safety*) por la EFSA, y/o de sus metabolitos antimicrobianos. El ejemplo más conocido son las bacterias lácticas, utilizadas en la elaboración de alimentos fermentados, que tienen la propiedad de actuar como conservantes naturales para mejorar la calidad y prolongar la vida útil de muchos alimentos. Otras sustancias, como lisozima, lactoperoxidasa, lactoferrina o aceites esenciales derivados de plantas, también presentan interés en alimentos para inhibir el desarrollo de microorganismos alterantes y patógenos.

4.2.1. Bacteriocinas

Las bacteriocinas son péptidos antimicrobianos de síntesis ribosomal producidos por bacterias, activos frente a bacterias relacionadas con la cepa productora. En general, son péptidos de bajo peso molecular, de naturaleza catiónica y anfipática y, en algunos casos, hidrofóbica, que suelen actuar sobre la membrana plasmática de las células sensibles. El mecanismo de acción de las bacteriocinas reside en la capacidad de formar poros en la membrana de la bacteria diana, lo que produce la disipación del potencial eléctrico transmembrana al promover la fuga de compuestos de bajo peso molecular, como iones, nucleótidos y aminoácidos, cuya pérdida conduce a la interrupción de los procesos celulares de biosíntesis, aunque también pueden actuar a nivel molecular (Bruno y Montville, 1993; Drider et al., 2006). Aunque la síntesis de bacteriocinas está muy ligada al crecimiento de la cepa productora, diversos factores como pH y temperatura juegan un papel importante en su producción, de tal forma que las condiciones favorables para el crecimiento de una cepa bacteriocinogénica podrían diferir de las óptimas para la producción de sus bacteriocinas (Aasen et al., 2000).

Las bacteriocinas producidas por las bacterias lácticas son particularmente interesantes para su empleo en el control de bacterias alterantes y/o patógenas en

alimentos. Su aplicación puede realizarse mediante la incorporación de la cepa productora como cultivo iniciador, adjunto o protector, o mediante la adición directa de la bacteriocina purificada o en forma de extracto semipurificado, como es el caso de la nisina, o de productos de la fermentación de otras cepas bacteriocinogénicas.

Las enterocinas son bacteriocinas producidas por bacterias lácticas pertenecientes al género *Enterococcus* (Khan et al., 2010) que en muchos casos son activas frente a *L. monocytogenes*. Entre ellas, se encuentran las enterocinas A y B, producidas por algunas cepas de *Enterococcus faecium*, que pertenecen a la clase IIa de la clasificación de bacteriocinas de Klaenhammer (1993), un grupo de péptidos lineales de menos de 10 kDa sin aminoácidos modificados. El mecanismo de acción de las bacteriocinas de clase IIa se basa en su acoplamiento a la manosa-fosfotransferasa, lo que conduce a la permeabilización de la membrana, formación de poros, interrupción de la fuerza motriz de protones y agotamiento de las reservas de ATP, que resulta a su vez en la detención de toda la biosíntesis celular (Bastos et al., 2015). Las enterocinas A y B actúan de forma sinérgica y complementaria, por lo que las bacterias resistentes a una de ellas podrían no serlo a la otra (Casaus et al., 1997). Las enterocinas A y B producidas por *E. faecium* CTC492 han demostrado ser efectivas frente a *L. monocytogenes* en jamón cocido, proporcionando protección extra cuando se rompe la cadena de frío (Garriga et al., 2002; Jofré et al., 2007b; Marcos et al., 2008). Sin embargo, se ha comprobado que algunos mecanismos, como una pared celular más cargada positivamente, una membrana celular más neutra y fluida y una expresión reducida de la manosa-fosfotransferasa pueden conferir resistencia a *L. monocytogenes* frente a las bacteriocinas de clase IIa (Vadyvaloo et al., 2004). Además, la eficacia de las bacteriocinas puede disminuir por distintos factores como adsorción a componentes, degradación por proteasas, baja solubilidad o distribución desigual en el alimento (Aasen et al., 2003).

4.2.2. Aceites esenciales

Los aceites esenciales (AEs) son mezclas naturales complejas resultantes del metabolismo secundario de las plantas que a menudo presentan propiedades antimicrobianas. Son considerados compuestos naturales por la industria alimentaria, empleándose para aumentar la vida útil de los productos, inhibiendo el crecimiento de microorganismos patógenos y alterantes en alimentos en sustitución de compuestos químicos (Raeisi et al., 2016; Tajkarimi et al., 2010). La actividad antimicrobiana de los AEs y sus componentes se debe a su capacidad de producir daños estructurales y

funcionales en la membrana celular de la bacteria diana (Tajkarimi et al., 2010), siendo generalmente las bacterias Gram-positivas más sensibles que las Gram-negativas (Kalemba y Kunicka, 2005). La capacidad anti-*Listeria* de distintos AEs, como el timol y su isómero el carvacrol, o de plantas ricas en ellos se ha investigado en productos cárnicos (Gouveia et al., 2017; Moon et al., 2017). El timol (2-isopropil-5-metilfenol) es el principal monoterpeno fenol que se encuentra en los AEs de plantas de la familia *Lamiaceae* así como en los géneros *Thymus*, *Ocimum*, *Origanum*, *Satureja*, *Thymbra*, y *Monarda* (Marchese et al., 2016). El timol y el carvacrol aumentan la permeabilidad de la membrana celular e inhiben la actividad de la ATP-asa con la consiguiente liberación de ATP intracelular y otros componentes celulares (Pisoschi et al., 2018). Algunos de los inconvenientes de la aplicación de estos compuestos son su volatilidad y olor, que podrían modificar las propiedades organolépticas del producto.

4.3. Tratamientos combinados

La teoría de barreras u obstáculos de Leistner (2000) supone la combinación de varios factores para el control microbiano. De acuerdo con esta teoría, las barreras más importantes para la conservación del jamón curado serían sus propias características intrínsecas como el pH y la baja a_w, producto de la adición de sales durante su elaboración. Aunque se ha demostrado la inactivación de *L. monocytogenes* en la superficie del jamón durante su maduración (Montiel et al., 2020), como se ha indicado anteriormente el producto podría contaminarse en las etapas de postprocesado. En este contexto, la combinación de varios tratamientos antimicrobianos podría tener un efecto sinérgico en la inactivación del patógeno, permitiendo reducir la intensidad de cada uno de ellos y disminuyendo así su impacto en el alimento.

La aplicación de las APH en combinación con distintos antimicrobianos ha dado buenos resultados en la inactivación de *L. monocytogenes* en productos cárnicos. Así, su combinación con lactato de sodio (Patterson et al., 2011) o lactoferrina (Del Olmo et al., 2012) incrementó el efecto inhibitorio frente a *L. monocytogenes* en carne de pollo. Aymerich et al. (2005) observaron que las células de *L. monocytogenes* supervivientes a las APH fueron más sensibles a nisina en jamón cocido. Del mismo modo, un tratamiento combinado de APH y enterocina LM-2 resultó muy efectivo en el control de *L. monocytogenes* en jamón cocido loncheado y envasado al vacío, prolongando su vida útil más de 90 días (Liu et al., 2012). La combinación con enterocinas A y B inhibió el crecimiento de este patógeno en embutidos fermentados, con recuentos inferiores a 1

unidad logarítmica tras 30 días de almacenamiento a temperatura ambiente (Jofré et al., 2009a), y en *carpaccio* de ternera curado, un tratamiento combinado de APH y enterocinas A y B o nisina tuvo un efecto anti-*Listeria* sinérgico tras 30 días de almacenamiento a 8 ºC (De Alba, 2014).

En el caso específico del jamón curado, la aplicación de nisina aumentó la inactivación de *L. monocytogenes* inducida por la alta presión, siendo mayor en muestras de jamón con a_w más alta (Hereu et al., 2012). También la combinación con enterocinas A y B, sakacina o nisina redujo los niveles del patógeno por debajo del límite de detección durante 120 días (Jofré et al., 2008). Asimismo, se observó un efecto sinérgico moderado al aplicar APH y lactoperoxidasa en jamón curado loncheado tras 60 días de almacenamiento a 8 ºC (De Alba et al., 2015).

La combinación de APH y AEs en la inactivación de *L. monocytogenes* apenas se ha estudiado en productos cárnicos. En jamón cocido loncheado, Pavli et al. (2019) investigaron el efecto del aceite esencial de orégano incorporado en películas comestibles de alginato de sodio en combinación con APH. La aplicación combinada consiguió reducir la carga microbiana en menor tiempo que los tratamientos aplicados individualmente. Respecto a otro tipo de productos, Bleoancă et al. (2016) observaron que la cinética de inactivación del patógeno en queso fresco se aceleraba al combinar las APH con un extracto de tomillo. En una bebida de yogur, la combinación con aceites esenciales de menta permitió disminuir la intensidad y el tiempo del tratamiento de APH, consiguiendo reducciones en los niveles de *L. monocytogenes* similares a las obtenidas con el tratamiento individual (Evrendilek y Balasubramanian, 2011). También se observó un efecto inhibitorio sinérgico frente a *L. monocytogenes* al combinar las APH con distintos AEs en medio de cultivo y leche (Karatzas et al., 2001) y en zumos de frutas (Espina et al., 2013).

El daño producido por las APH en las membranas celulares de las bacterias facilitaría la acción de los antimicrobianos potenciando su efecto. Aunque los estudios de aplicación de tratamientos combinados en jamón curado son escasos, su uso podría resultar una herramienta eficaz para controlar *L. monocytogenes* en este producto.

5. Impacto de los tratamientos antimicrobianos en la virulencia de *L. monocytogenes*

Los tratamientos de presurización y bioconservación han demostrado su eficacia en el control de *L. monocytogenes* en alimentos, pero apenas se conoce su efecto en las células que sobreviven a los mismos. La exposición a condiciones de estrés subletales

puede derivar en la adaptación del patógeno a condiciones de estrés letales o producir cambios en su expresión génica (Garner et al., 2006). Así, se ha estudiado el efecto de tratamientos de APH en la expresión génica de *L. monocytogenes* en medio de cultivo (Bowman et al., 2008) y de las bacteriocinas en medio de cultivo y leche (Miranda et al., 2018; Ye et al., 2017). Sin embargo, no se ha investigado cómo afectan estos tratamientos a la fisiología o la patogenicidad de *L. monocytogenes* en jamón curado. Una de las mejores aproximaciones para ampliar dicho conocimiento es el estudio de la expresión de genes relacionados con la virulencia y la resistencia al estrés.

El método más común para analizar la expresión génica consiste en realizar una transcripción reversa seguida de la reacción en cadena de la polimerasa a tiempo real (RT-qPCR). Es un método sensible, preciso, reproducible y rápido, aunque su aplicación puede complicarse debido a los posibles inhibidores de PCR presentes en matrices alimentarias (Auvolat y Besse, 2016). Esta técnica consta de tres etapas (Figura 6): síntesis de ADN complementario (ADNc) a partir de ARN mensajero (ARNm) obtenido de la muestra problema utilizando una transcriptasa inversa (A); amplificación del ADNc por qPCR (B) y análisis de los datos obtenidos (C).

La detección en tiempo real es posible gracias al uso de compuestos que emiten fluorescencia a medida que se va produciendo la amplificación, estando dicha fluorescencia directamente relacionada con la cantidad de ácido nucleico de partida. Existen muchos tipos de fluoróforos, entre los que destacan el SYBR Green, compuesto que emite fluorescencia cuando se une inespecíficamente a ADN de doble cadena, y las sondas de hidrólisis como las sondas TaqMan. Estas sondas se diseñan específicamente para el gen diana y se basan en la actividad exonucleasa 5'-3' de la polimerasa, que hidroliza la sonda cuando la alcanza durante la extensión y permite así la emisión de fluorescencia por parte de la molécula "reportera", fluorescencia que inicialmente estaba bloqueada por la acción de otra molécula "supresora o *quencher*" presente en la sonda. En cualquier caso, el principio de la técnica se basa en detectar el ciclo de amplificación en que se ha generado suficiente producto de PCR como para poder distinguir su fluorescencia de la del ruido de fondo, el cual se denomina ciclo umbral o C_T (Stephenson, 2010).

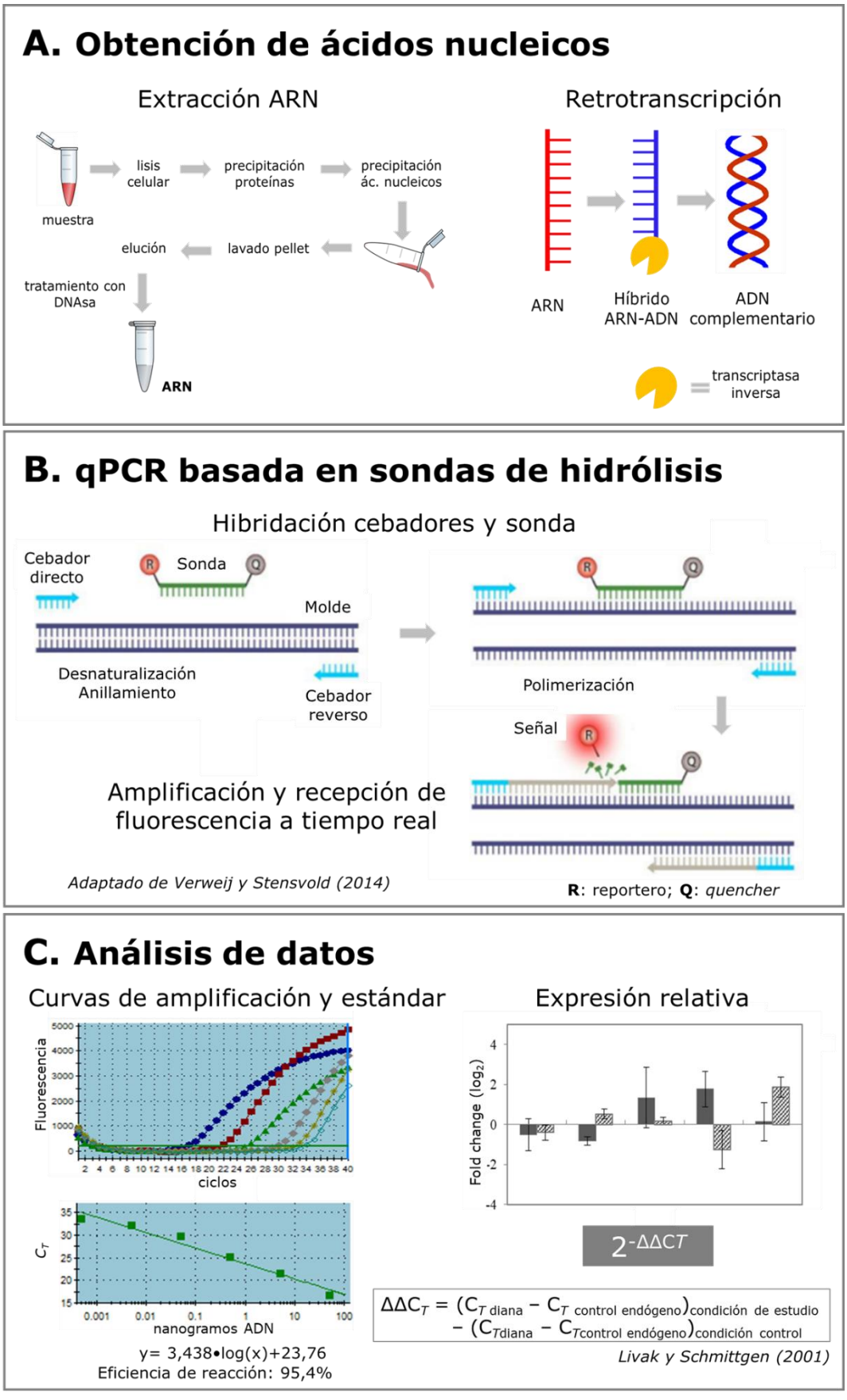

Figura 6. Representación esquemática del análisis de la expresión génica basado en sondas de hidrólisis (metodología TaqMan).

El estudio de la expresión génica puede ser cuantitativo, que determina el número de copias de partida del transcrito de interés mediante el uso de curvas estándar construidas con concentraciones de partida conocidas, o relativo. La expresión génica relativa es la más utilizada para estudiar cambios fisiológicos mediante la comparación de la expresión de un gen diana bajo una condición de estudio con respecto a su expresión en una condición control. Este tipo de análisis requiere el uso de uno o varios genes de referencia (controles endógenos), que son genes constitutivos de las células cuya expresión no varía. Así, suelen utilizarse como controles endógenos los genes codificantes de la subunidad 16S del ARNr (Karatzas et al., 2010), de la subunidad beta de la ARN polimerasa dependiente de ADN (*rpoB*) (Mataragas et al., 2014; Olesen et al., 2010) o la región separadora intergénica (IGS) (Alía et al., 2019; Rantsiou et al., 2012) porque su expresión es relativamente constante. El propósito de utilizar un control endógeno es normalizar la cantidad de ARNm añadido para la transcripción inversa, así como compensar las variaciones inter-PCR (Pfaffl, 2001). Existen varios modelos matemáticos para calcular la expresión relativa, siendo el método $2^{-\Delta\Delta CT}$ uno de los más empleados, donde $\Delta\Delta C_T$ es: $(C_{T\ \text{diana}} - C_{T\ \text{control endógeno}})_{\text{condición de estudio}} - (C_{T\text{diana}} - C_{T\text{control endógeno}})_{\text{condición control}}$ (Livak y Schmittgen, 2001). Este método requiere validar la elección del gen endógeno para asegurar que la condición de estudio no modifica su expresión y que se pueda amplificar con una eficiencia similar a la del gen diana.

Objetivos

El objetivo general de esta tesis doctoral consiste en mejorar la seguridad microbiológica del jamón curado mediante la investigación de la contaminación de *L. monocytogenes* en industrias productoras, la aplicación de distintas estrategias de inactivación del patógeno y el análisis de su impacto en la virulencia y resistencia a estrés de *L. monocytogenes*.

Para ello se han planteado los siguientes objetivos parciales:

Objetivo 1: Caracterización de la contaminación por *L. monocytogenes* en instalaciones y equipos de plantas de procesado de jamón curado.

Objetivo 2: Aplicación de altas presiones y bioconservación frente a *L. monocytogenes* en jamón curado.

Objetivo 3: Evaluación del efecto de los tratamientos antimicrobianos en la expresión de genes de virulencia y de respuesta a estrés de *L. monocytogenes*.

La sección de resultados de esta tesis doctoral se ha dividido en tres capítulos, cada uno de los cuales aborda uno de los objetivos específicos planteados.

Resultados

Capítulo 1

Caracterización de la contaminación por *L. monocytogenes* en plantas de procesado de jamón curado

L. monocytogenes es una bacteria ubicua que está presente en las instalaciones y maquinaria de las plantas de producción de distintos alimentos, y uno de los patógenos que más preocupa a la industria cárnica. Por ello, el primer objetivo de este trabajo fue investigar la presencia de *L. monocytogenes* en el ambiente y equipos de las zonas de deshuesado y loncheado de tres empresas españolas productoras de jamón curado. Se ha analizado la diversidad genética de los aislados obtenidos, así como la virulencia, la resistencia potencial a desinfectantes y la formación de biofilms. Los resultados obtenidos se recogen en una publicación científica incluida en el *Science Citation Index* (SCI):

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Genomic diversity and characterization of *Listeria monocytogenes* from dry-cured ham processing plants

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ABSTRACT

Genomic diversity of *Listeria monocytogenes* isolates from the deboning and slicing areas of three dry-cured ham processing plants was analysed. *L. monocytogenes* was detected in 58 out of 491 samples from the environment and equipment surfaces, all from the deboning area, with differences in prevalence among facilities. The most frequent PCR-serogroup was IIa (74.1%) followed by IIb and IIc, and only one isolate was serogroup IVb. Twenty different pulsotypes and 11 sequence types (STs) grouped into 10 clonal complexes (CCs) were determined. ST121 (CC121) and ST9 (CC9) were the most abundant. Premature stop codons (PMSC6 and PMSC19) associated with attenuated virulence were found in the *inlA* sequence in 7 out of 12 selected strains. CC121 strains were strong biofilm formers and some harboured the transposon Tn*6188*, related with increased tolerance to quaternary ammonium compounds. *L. monocytogenes* clones considered hypovirulent resulted predominant in the deboning areas. The clonal structure and potential virulence of the isolates could help to establish adequate control measures and cleaning protocols for the comprehensive elimination of the pathogen in dry-cured ham processing environment.

1. Introduction

Listeria monocytogenes is a foodborne pathogen that causes listeriosis, which affects mainly the elderly, immunocompromised individuals, pregnant women and new-borns. It is the most severe foodborne disease in terms of hospitalization (more than 95% of cases) and has the highest mortality rate in the European Union, accounting for 15.6% in 2018 (EFSA and ECDC, 2019). Many outbreaks of listeriosis have been reported after consumption of meat products in different countries, including the USA (Olsen et al., 2005), New Zealand (Rivas et al., 2019), South Africa (Thomas et al., 2020) or Spain (WHO, 2019). Listeriosis is mainly associated with the consumption of ready-to-eat (RTE) food products contaminated with the pathogen, being RTE meat products the cause of the majority of deaths associated with food and drink consumption during 2018 in EU (EFSA and ECDC, 2019). Food safety regulations in the European Union established a maximum of 100 CFU/g of *L. monocytogenes* for RTE products which cannot support the growth of the pathogen during their shelf-life and that are not intended for infants and medical purposes (EC, 2005), while a zero-tolerance (absence in 25 g) is required for this pathogen in the USA (USDA-FSIS, 2014).

L. monocytogenes is commonly found in different environments, including soil, sewage, freshwater and many raw foods and it has been reported to enter in processing plant facilities from multiple sources, including raw material, machinery and operators (Ricci et al., 2018). Once inside, certain strains could be established in the food processing installations and survive for a long time due to their ability to withstand environmental stresses and to grow at refrigeration temperatures (<5 °C), low pH (>4.4) and high salt concentration (up to 12%) (Lado and Yousef, 2007). Among the 14 serotypes of *L. monocytogenes* identified, grouped into 4 lineages, serotypes 4b and 1/2a predominate in cases of human listeriosis and in the food environment, respectively (Orsi et al., 2011; Poimenidou et al., 2018; Yin et al., 2019). Some of these strains linked to food processing environments exhibit mutations in virulence genes that produce truncated proteins, including PrfA, the major virulence regulator of *L. monocytogenes* (Sheehan et al., 1995), and InlA, an internalin essential for the pathogen to invade intestinal cells (Gaillard et al., 1991). Further, *L. monocytogenes* is able to form biofilm by adhering to abiotic surfaces widely used in the food industry and to tolerate commonly used disinfectants which contributes to the pathogen persistence for several months or years (Lee et al., 2019; Stoller et al., 2019). *L. monocytogenes* tolerance to disinfectants may be due to the presence of sub-inhibitory concentrations of disinfectant, to

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genetic mutations that would reduce the permeability of the pathogen membrane to such compounds, or to the presence of resistance genes such as the *qacH* gene of transposon Tn*6188* or the resistance determinant *bcrABC* (Martínez-Suárez et al., 2016; Müller et al., 2013, 2014; Elhanafi et al., 2010).

Total control of the pathogen in processing plants, including non-food contact surfaces, is extremely difficult (Carpentier and Cerf, 2011). Samples taken from different food facilities (dairy, meat, vegetable and others) in the Republic of Ireland revealed the presence of *L. monocytogenes* in 62% of the installations and the evidence of bacterial transfer from the processing environment to the food products (Leong et al., 2014). Different molecular typing methods have been employed to assess the source of contamination and the routes of transmission of *L. monocytogenes*. PCR-serogrouping is used for a rapid discrimination of *L. monocytogenes*. Pulsed-field gel electrophoresis (PFGE), used to differentiate *L. monocytogenes* isolates by their unique banding patterns, and methods based on DNA sequence, such as multilocus sequence typing (MLST) or complete sequencing genome (WGS), proved to be particularly suitable for studies on the epidemiology of *L. monocytogenes* (Henri et al., 2016, 2017).

Dry-cured ham is considered as a safe product due to its low water activity and high salt content. The inactivation of *L. monocytogenes* during dry-cured ham processing has been demonstrated (Montiel et al., 2020) but post-processing contamination may lead to the presence of the pathogen in the final product, compromising the fulfilment of legal or commercial requirements, as the zero-tolerance policies. Therefore, the objective of this work was to investigate the presence of *L. monocytogenes* in the deboning and slicing areas of three dry-cured ham processing plants and to determine the clonal complexes, the variation of virulence genes, the detection of genetic markers of resistance to disinfectants and the biofilm formation of *L. monocytogenes* isolates. The analysis of such characteristics would increase the knowledge of *L. monocytogenes* strains present in dry-cured ham plants and contribute to improve strategies to eliminate the pathogen.

2. Materials and methods

2.1. Sampling procedure

Environmental and equipment surfaces from three dry-cured ham processing plants (A, B and C) located in different Spanish provinces were sampled to detect *L. monocytogenes*. Sample collection was performed during production process (DPP) and before production process (BPP, after cleaning and disinfection procedures), from food contact equipment surfaces (FCS) and from environment and non-food contact surfaces (NFCS) at the deboning and slicing areas. A total of 491 samples were collected, 246 DPP and 245 BPP, corresponding to 309 from FCS and 182 from NFCS. Sampling period varied between 1 and 3 years depending on the facility. Pre-moistened sterile wipes (bioMérieux España SAU, Madrid, Spain) were used for surface sampling in both horizontal and vertical directions, put into sterile stomacher bags and kept in a cooler until the analysis.

2.2. L. monocytogenes isolation

USDA-FSIS protocol was used for the detection of *L. monocytogenes* (USDA-FSIS, 2013) with some modifications here described. Samples were homogenized with 225 mL of UVM Modified *Listeria* Enrichment Broth (UVM-I, Conda Lab S.A., Madrid, Spain) and incubated for 24 h at 30 °C. Primary enrichment broth (100 μL) was transferred to 10 mL of Fraser Broth (Conda Lab) which was incubated at 37 °C for 48 h. Primary and secondary enrichment broths were streaked on duplicate plates of CHROMagar *Listeria* (CH-L, Scharlab S.L., Barcelona, Spain) which were examined after incubation at 37 °C up to 48 h for presence of the pathogen. Isolates were maintained at −80 °C in Brain Heart Infusion broth (BHI, Biolife S.r.l., Milan, Italy) with 20% glycerol. Before use in experiments, isolates were subcultured twice in Tryptic Soy Yeast Extract Broth (TSYEB, Biolife) at 37 °C for 18 h.

2.3. PCR-serogrouping

A rapid extraction of total DNA was performed from pure *L. monocytogenes* cultures in Tryptic Soy Yeast Extract Agar (TSYEA, Biolife) plates incubated at 37 °C for 24 h. Several colonies were resuspended in a mixture of 0.25% sodium dodecyl sulfate (SDS, Panreac Química SLU, Barcelona, Spain) and 0.05 M sodium hydroxide (NaOH, Panreac). Lysis was performed by heat and, after centrifugation, the supernatants containing DNA were recovered and stored at −20 °C until use. *L. monocytogenes* isolates were confirmed by PCR-serogrouping with a multiplex PCR assay (Doumith et al., 2004), which differentiated the major groups (IIa: 1/2a-3a, IIb: 1/2b-3b-7, IIc: 1/2c-3c, IVb: 4b-4d-4e and IVb-v1: 4b) by targeting the marker genes *lmo0737*, *lmo1118*, ORF2819 and ORF2110 and the *prs* gene (Table 1).

2.4. Pulsed-field gel electrophoresis (PFGE) typing

The *L. monocytogenes* DNA fingerprint was determined by PFGE according to the Centers for Disease Control and Prevention PulseNet standardized procedure (Halpin et al., 2010). DNA digest was performed using the restriction enzymes AscI and ApaI (New England Biolabs Inc., Ipswich, MA, USA). The PFGE global standard *Salmonella* ser. Braenderup H9812 digested with XbaI (New England Biolabs Inc.) was used in the analysis. The restriction DNA fragments were separated by using the polygonal contour clamped homogeneous electric field system CHEF DRII (Bio-Rad Laboratories, Hercules, CA, USA). AscI/ApaI macrorestriction patterns were analysed using the BioNumerics software package 7.1 (Applied Maths NV, Sint-Martens-Latem, Belgium). PFGE patterns (pulsotypes, PTs) were compared by calculation of the Dice correlation coefficient with optimization and tolerance of 1.5 and were clustered into a dendrogram by the unweighted pair group method with arithmetic mean (UPGMA) method.

2.5. Multilocus sequence typing (MLST)

MLST analysis was carried out based on the sequence analysis of the following seven housekeeping genes: *abcZ*, *bglA*, *cat*, *dapE*, *dat*, *ldh* and *lhkA* (Table 1) (Ragon et al., 2008). Each 20 μL-PCR reaction contained 200 μM of each primer and 1 μL of extracted DNA from *L. monocytogenes* isolates. Amplification conditions were as follows: an initial cycle of 94 °C for 4 min; 25 amplification cycles, each consisting of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 2 min; and a final incubation at 72 °C for 10 min. PCR products were purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific, Whaltman, MA, USA) and sequenced by the Sanger Sequencing Service (Complutense University of Madrid, Spain). Multilocus sequence types (STs) and clonal complexes (CCs) were assigned using the *Listeria* MLST database at the Pasteur Institute website (http://www.pasteur.fr/mlst). The analysis of the genetic relationship among the different STs was investigated using the goeBURST algorithm, with Phyloviz software (http://phyloviz.net). A minimum spanning tree (MST) representation of this relationship separated the STs that differed in one or more alleles.

2.6. Virulence and persistence of L. monocytogenes

Among *L. monocytogenes* isolated from environment and equipment surfaces, 12 strains were selected to determine the variation of virulence genes, the detection of genetic markers of resistance to disinfectants and the biofilm formation.

2.6.1. Genetic analysis of virulence

The 2400 bp long *inlA* gene and a 469 bp *prfA* internal fragment from *L. monocytogenes* strains were sequenced in this study. PCR amplification

Table 1
PCR primers used in this study.

	Gene	Function and scope of use	Sequence (5′ → 3′)	Amplicon size (bp)	Reference
Serogrouping	*lmo0737*	Uncharacterized protein	F: AGGGCTTCAAGGACTTACCC R: ACGATTTCTGCTTGCCATTC	691	Doumith et al. (2004)
	lmo1118	Uncharacterized protein	F: AGGGGTCTTAAATCCTGGAA R: CGGCTTGTTCGGCATACTTA	906	Doumith et al. (2004)
	ORF2819	Putative transcriptional regulator	F: AGCAAAATGCCAAAACTCGT R: CATCACTAAAGCCTCCCATTG	471	Doumith et al. (2004)
	ORF2110	Putative secreted protein	F: AGTGGACAATTGATTGGTGAA R: CATCCATCCCTTACTTTGGAC	597	Doumith et al. (2004)
	prs	Putative phosphoribosyl pyrophosphate synthetase	F: GCTGAAGAGATTGCGAAAGAAG R: CAAAGAAACCTTGGATTTGCGG	370	Doumith et al. (2004)
MLST	*abcZ*	ABC transporter	F: TCGCTGCTGCCACTTTTATCCA R: CTCAAGGTCGCCGTTTAGAG	537	http://www.pasteur.fr/mlst
	bglA	Beta-glucosidase	F: GCCGACTTTTTATGGGGTGGAG R: CCGATTAAATACGGTGCGGACATA	399	http://www.pasteur.fr/mlst
	cat	Catalase	F: ATTGGCGCATTTTGATAGAGA R: CAGATTGACGATTCCTGCTTTTG	486	http://www.pasteur.fr/mlst
	dapE	Succinyl diaminopimelate desuccinylase	F: CGACTAATGGGCATGAAGAACAAG R: CATCGAACTATGGGCATTTTTACC	462	http://www.pasteur.fr/mlst
	dat	D-amino acid aminotransferase	F: GAAAGAGAAGATGCCACAGTTGA R: CTGCGTCCATAATACACCATCTTT	471	http://www.pasteur.fr/mlst
	ldh	Lactate dehydrogenase	F: CGACGGAGCAGTTGGTTCTA R: AACGCCTTGACGGTTAACGA	802	This study
	lhkA	Histidine kinase	F: AGAATGCCAACGACGAAACC R: CTGGGAAACATCAGCAATAAAC	480	http://www.pasteur.fr/mlst
Virulence	*inlA*	Invasion of intestinal epithelial cells	F0: CGGATGCAGGAGAAAATCC R0: CTTTCACACTATCCTCTCC F1: GATATAACTCCACTTGGG R1: GCTCTAAGTTAGTGAGTGCG F2: GTGGACGGCAAAGAAAC R2: GAGATGTTGTTACACCGTC	2400	Ragon et al. (2008)
	prfA	Virulence transcription regulator	F: AACGGGATAAAACCAAAACCA R: TGCGATGCCACTTGAATATC	469	Zhang et al. (2004)
QACs resistance[a]	*qacH*	Transporter putatively associated with export of BC[b]	F: ATGTCATATCTATATTTAGC R: TCACTCTTCATTAATTGTAATAG	366	Müller et al. (2013)
	radC	DNA repair protein	F: CTTGCCAATGATAATATGATC R: GTGGTCTGAATGCTCCATCG	200[c] 5310[d]	Müller et al. (2013)
	bcrABC	BC resistance cassette	F: GGAGGGTAATCATGTCAG R: GTATAATCCGGATGCTGCCC	1312	Elhanafi et al. (2010)

[a] quaternary ammonium compounds.
[b] benzalkonium chloride.
[c] amplicon size (bp) of a chromosomal *L. monocytogenes radC* gene fragment.
[d] amplicon size (bp) of a chromosomal *L. monocytogenes radC* gene fragment when the Tn*6188* transposon (which carries the *qacH* gene) has been integrated.

was performed as described Ragon et al. (2008) and Zhang et al. (2004), respectively. PCR products were purified and sequenced as indicated above. Primers used are detailed in Table 1. Sequence analysis was performed using the BIOEDIT Sequence Alignment Editor 7.0.9 (Ibis Biosciences, Carlsbad, CA, USA). The sequencing products were edited and compared with the *inlA* or *prfA* sequences of *L. monocytogenes* reference strain EGDe, available in the GenBank database (NC_003210.1) by manual alignment using the ClustalW algorithm (Thompson et al., 1994). The nucleotide sequences obtained were translated into their corresponding amino acid sequences by the nucleotide translate application ExPASy (Swiss Institute of Bioinformatics, Laussane, Switzerland). Subsequently, the amino acid sequences were aligned by ClustalW and analysed with GeneDoc 2.7 software (Nicholas et al., 1997) to identify changes in the compositions of their respective proteins, which might modify or eliminate protein functions.

2.6.2. Detection of genetic markers of resistance to disinfectants

The potential tolerance of *L. monocytogenes* strains to disinfectants was analysed by amplification of the *qacH*, *radC* and *bcrABC* genes (Table 1). The amplification of the *qacH*, a gene from the transposon Tn*6188* responsible for tolerance to QACs (quaternary ammonium compounds), including benzalkonium chloride (BC), and the flanking *radC* gene, into which the Tn*6188* is integrated, was performed as described by Müller et al. (2013). The BC resistance cassette (*bcrABC*) plasmid-associated was amplified as described by Elhanafi et al. (2010). *L. monocytogenes* 4423 and CDL69 were included as positive controls for the *qacH* and *bcrABC*, respectively (Müller et al., 2013).

2.6.3. Biofilm formation

The biofilm forming ability of *L. monocytogenes* strains was investigated in 100-well microplates Honeycomb (Bio-Rad Laboratories) following the method described by Pan et al. (2010) with some modifications. Plates were inoculated with 10^6 CFU/mL of each strain and incubated for 4 d at 22 °C or for 7 d at 8 °C as described by Stoller et al. (2019). Biofilms were washed five times with sterile distilled water, stained with crystal violet (0.8%), washed five times with distilled water and air dried. Ethanol (95%) was used to destain the biofilms and the OD_{580nm} was measured in an automated spectrophotometer (Bioscreen C, Bio-Rad Laboratories). *L. monocytogenes* Scott A (biofilm forming strain) was used as positive control and sterile media as negative control. Two experiments with triplicate samples were performed for each temperature from independently grown bacterial cultures. Data from the absorbance of individual strains were analysed using box plots.

3. Results and discussion

3.1. Prevalence of L. monocytogenes in the environment and equipment surfaces

A total of 491 samples obtained from the environment and

equipment surfaces at the deboning and slicing areas of three dry-cured ham processing facilities were analysed (Table 2). Of these, 11.8% were positive for *L. monocytogenes*, all from the deboning areas. *L. monocytogenes* was found in all the processing facilities investigated. Higher prevalence was registered in plant B (23.9%), followed by plant C (9.9%) and plant A (8.0%). In a similar study, Alía et al. (2020) detected *L. monocytogenes* in five out of six dry-cured ham plants, with an overall incidence of 9.2% of the surfaces analysed from the deboning and slicing areas. The presence of the pathogen in the deboning areas could be attributed to the invasive mechanical operations required to extract the bone before slicing, with a higher chance of pathogen introduction and spreading on the equipment, floor and handler's clothing. Contamination patterns in Italian cured ham processing facilities revealed higher prevalence of the pathogen at the initial processing steps, decreasing from post-salting to the end of curing (Morganti et al., 2016). According to these authors, prevalence increased from 6.5% in ham processing areas to 10.1% at deboning.

L. monocytogenes was detected in 38 (7.7%) surfaces sampled during processing (DPP) and in 20 (4.1%) cleaned and disinfected surfaces before processing (BPP). The percentage of positive samples diminished after cleaning and sanitizing procedures, which was 2.7, 11.4 and 2.4% for Plants A, B and C, respectively, differences probably attributable to variations in cleaning practices. More than 50% of these positive samples were taken from non-food contact surfaces. In the same way, data on the presence of *L. monocytogenes* in the environment and equipment of pig processing plants after cleaning and disinfection revealed values around 10.0% (D'Arrigo et al., 2020; Ortiz et al., 2010). Contamination of meat products in processing plants is mostly related with strains already present in the plant environment (López et al., 2008). The presence of *L. monocytogenes* in the final stages of processing represents a potential risk for cross-contamination of the finished product (Sereno et al., 2019). Poorly maintained equipment and surfaces during the manufacture of dry-cured ham can protect bacteria from cleaning and sanitizing procedures allowing *L. monocytogenes* niches or harbourage sites in the processing environment. Further, washing with low pressure hoses or high pressure jets could spread the bacteria in liquids or aerosols. In the present work, 34.5% of positive samples was obtained after cleaning and disinfection. This persistence of the pathogen could be linked to some properties of the bacterium as the capacity to form biofilms and/or the high natural tolerance to biocides (Orsi et al., 2011). Maury et al. (2019) concluded that the use of low concentrations of benzalkonium chloride (BC) during the cleaning and disinfection procedures in the meat industry could lead to the appearance of persisting clones highly adapted to the environment owing to efficient biofilm formation.

3.2. Characterization of *L. monocytogenes* isolates

Four PCR-serogroups, IIa, IIb, IIc and IVb, were detected among the 58 *L. monocytogenes* isolates from the environment and equipment surfaces of the three processing facilities (Table 3). The most common was IIa (74.1%), followed by IIb and IIc (12.1%). Only one isolate of *L. monocytogenes* was ascribed to PCR-serogroup IVb, collected in plant B. Among the four evolutionary lineages and the 14 serotypes of *L. monocytogenes* described, serotypes 1/2a, 1/2b and 4b cause more than 95% of human cases of listeriosis (Kathariou, 2002). Serotype 4b (lineage I) is most frequently associated with outbreaks and sporadic cases, while strains belonging to lineage II (1/2a and 1/2c) are related with food or food processing environment (Orsi et al., 2011). In the present work, PCR-serogroup IIa predominated in all facilities analysed, representing 75.0, 47.6 and 96.0% of the isolates from plants A, B and C, respectively. *L. monocytogenes* IVb isolate, registered in plant B, was recovered from a drain during processing, but was not re-isolated after cleaning and disinfection. Serotype 1/2a has been associated with meat and meat processing environments (López et al., 2008; Martín et al., 2014; Ortiz et al., 2010), where serotype 4b was less abundant (Alía et al., 2020; Martín et al., 2014; Stoller et al., 2019).

The combined analysis of the AscI and ApaI profiles allowed the classification of the 58 isolates into 20 different pulsotypes (PTs) (Fig. 1). Four PTs were obtained from Plant A. Pulsotype A2 was the most common (58.3%), found during processing and after cleaning and disinfection and from food contact and non-food contact surfaces. PTs A1, A3 and A4 were only detected during processing. In plant B, five different PTs were obtained. The predominant pulsotype B4 (47.6%) was isolated during and before processing and from food contact and non-food contact surfaces in several samples taken over 2 years, pointing out to its persistence in the environment of this facility. PTs B1 and B5 were also isolated from dirty and clean areas. The highest diversity was found in Plant C, with 11 different PTs. The most common was pulsotype C1 (20.0%) followed by C3 and C5 (both with 16.0% of the isolates). These PTs were repeatedly isolated before and after cleaning and disinfection over time. Several sporadic PTs were found in all processing plants, whereas common PTs were not registered among *L. monocytogenes* isolated from the three different plants (Table 3).

L. monocytogenes PTs were considered persistent when they were found repeatedly (three times or more) in environmental or equipment surfaces over a minimum of a 3-month period (Keto-Timonen et al., 2007). In this study, four PTs were considered persistent, one PT in plant B (B4) and three in plant C (C1, C3 and C5). B4 was isolated from FCS, such as deboning table and cleaning table before pressing machine, and from NFCS, including deboning room wall and drains. C1 was found in the pressing mold and peeling table (FCS) and in a room drain (NFCS). C3 was recovered only from NFCS (a room drain or trays for tools) and C5 from peeling tools (FCS) and a room drain and floor (NFCS). These results are in agreement with previous published data. In general, a low number of persistent PTs is found in food processing environments, but sporadic incidence of *L. monocytogenes* with relative high strain diversity has been reported (Alía et al., 2020; Bolocan et al., 2016; Ortiz et al., 2010). Although the same PFGE pattern was not recorded among the three different plants investigated, genetically similar PTs were identified, suggesting that certain strains could be adapted to the environment of meat processing facilities.

Allelic profiles obtained by MLST allowed the definition of 11 different sequence types (STs), belonging to 10 different clonal complexes (CCs) (Table 3). The most abundant genotypes were ST121 (48.3%) followed by ST9 (12.1%), both from lineage II and detected in the three plants. ST87 (8.6%) and ST1, ST3, ST8, ST31, ST37, ST155, ST236 and ST504 (less than 7%) were less abundant and only found in one of the plants. PCR-serogroup IIa registered the highest diversity, with seven STs (ST8, ST31, ST37, ST121, ST155, ST236 and ST504). Molecular serogroup IIb isolates were typed as ST3 and ST87, all IIc isolates were ST9 whereas the only IVb strain corresponded to ST1 (Fig. 2).

L. monocytogenes ST121 (CC121) and ST9 (CC9) were the most prevalent among food strains in France, being ST121 the most common in number and diversity of food matrices, whereas represented only 1% of clinical isolates (Henri et al., 2016). *L. monocytogenes* ST9 was

Table 2
Prevalence of *L. monocytogenes* in the environment and equipment surfaces from the deboning and slicing areas of three dry-cured ham processing facilities.

	No. samples	No. positive	DPP[a] (No. positive/No. samples)		BPP[b] (No. positive/No. samples)	
			FCS[c]	NFCS[d]	FCS	NFCS
Plant A	150	12	4/43	4/26	2/55	2/26
Plant B	88	21	5/22	6/22	6/21	4/23
Plant C	253	25	10/86	9/47	1/82	5/38

[a] during production process.
[b] before production process.
[c] food contact surfaces.
[d] non-food contact surfaces.

Table 3
Molecular subtypes of *L. monocytogenes* identified in this study.

Isolate	Plant	Sample	DPP[a]/BPP[b]	FCS[c]/NFCS[d]	Lineage	PCR-serogroup	Pulsotype	Sequence Type	Clonal Complex
A001[e]	A	Saw	DPP	FCS	II	IIa	A2	ST121	CC121
A002	A	Saw	DPP	FCS	II	IIa	A2	ST121	CC121
A003	A	Conveyor belt	DPP	FCS	II	IIa	A2	ST121	CC121
A004	A	Moulding machine	DPP	NFCS	I	IIb	A3	ST3	CC3
A005	A	Mold packaging machine	DPP	NFCS	I	IIb	A3	ST3	CC3
A006	A	Conveyor belt	DPP	NFCS	II	IIa	A1	ST121	CC121
A007[e]	A	Mold packaging machine	DPP	FCS	II	IIc	A4	ST9	CC9
A008	A	Drain	DPP	NFCS	II	IIa	A1	ST121	CC121
A009[e]	A	Saw	BPP	FCS	II	IIa	A2	ST121	CC121
A010	A	Saw	BPP	NFCS	II	IIa	A2	ST121	CC121
A011	A	Gouge table	BPP	NFCS	II	IIa	A2	ST121	CC121
A012	A	Gouge	BPP	FCS	II	IIa	A2	ST121	CC121
B001[e]	B	Container	DPP	FCS	II	IIc	B5	ST9	CC9
B002[e]	B	Deboning table	DPP	FCS	I	IIb	B1	ST87	CC87
B003	B	Cleaning table	DPP	FCS	II	IIa	B4	ST121	CC121
B004	B	Drain	DPP	NFCS	II	IIc	B5	ST9	CC9
B005	B	Trash can	DPP	NFCS	II	IIc	B5	ST9	CC9
B006	B	Floor	DPP	NFCS	I	IIb	B1	ST87	CC87
B007[e]	B	Deboning table	BPP	FCS	II	IIa	B4	ST121	CC121
B008	B	Deboning table	BPP	FCS	II	IIc	B5	ST9	CC9
B009	B	Cleaning table	BPP	FCS	II	IIa	B4	ST121	CC121
B010	B	Cleaning table	BPP	FCS	II	IIa	B4	ST121	CC121
B011	B	Floor	BPP	NFCS	II	IIc	B5	ST9	CC9
B012[e]	B	Drain	BPP	NFCS	I	IIb	B2	ST87	CC87
B013	B	Drain	DPP	NFCS	II	IIa	B4	ST121	CC121
B014	B	Deboning table	DPP	FCS	II	IIa	B4	ST121	CC121
B015	B	Drain	DPP	NFCS	II	IIa	B4	ST121	CC121
B016	B	Deboning table	BPP	FCS	II	IIa	B4	ST121	CC121
B017	B	Drain	BPP	NFCS	II	IIa	B4	ST121	CC121
B018	B	Deboning table	DPP	FCS	I	IIb	B1	ST87	CC87
B019	B	Deboning room wall	BPP	NFCS	II	IIa	B4	ST121	CC121
B020	B	Deboning table	BPP	FCS	I	IIb	B1	ST87	CC87
B021[e]	B	Drain	DPP	NFCS	I	IVb	B3	ST1	CC1
C001	C	Tray for tools	DPP	NFCS	II	IIa	C3	ST236	CC121
C002[e]	C	Floor	DPP	NFCS	II	IIa	C1	ST121	CC121
C003	C	Peeling tools	DPP	FCS	II	IIa	C5	ST504	CC475
C004	C	Pressing machine	DPP	FCS	II	IIa	C6	ST8	CC8
C005	C	Pressing mold	DPP	FCS	II	IIa	C1	ST121	CC121
C006	C	Shelving wheels	DPP	NFCS	II	IIa	C5	ST504	CC475
C007	C	Deboning tools	DPP	FCS	II	IIa	C10	ST155	CC155
C008	C	Tray for tools	DPP	NFCS	II	IIa	C3	ST236	CC121
C009	C	Conveyor belt	DPP	FCS	II	IIa	C8	ST31	CC31
C010	C	Shelving wheels	DPP	NFCS	II	IIa	C7	ST8	CC8
C011	C	Floor	DPP	NFCS	II	IIa	C5	ST504	CC475
C012	C	Drain	DPP	NFCS	II	IIa	C1	ST121	CC121
C013	C	Peeling table	DPP	FCS	II	IIa	C1	ST121	CC121
C014	C	Drain	DPP	NFCS	II	IIa	C3	ST236	CC121
C015[e]	C	Pressing machine	DPP	FCS	II	IIa	C2	ST121	CC121
C016	C	Tray for tools	BPP	NFCS	II	IIa	C3	ST236	CC121
C017	C	Mold packaging machine	BPP	NFCS	II	IIa	C1	ST121	CC121
C018	C	Drain	BPP	NFCS	II	IIa	C5	ST504	CC475
C019	C	Shelving wheels	BPP	NFCS	II	IIa	C8	ST31	CC31
C020	C	Drain	BPP	NFCS	II	IIa	C8	ST31	CC31
C021[e]	C	Conveyor belt	DPP	FCS	II	IIa	C4	ST121	CC121
C022[e]	C	Deboning table	DPP	FCS	II	IIc	C9	ST9	CC9
C023	C	Deboning tools	DPP	FCS	II	IIa	C4	ST121	CC121
C024	C	Peeling belt	DPP	NFCS	II	IIa	C4	ST121	CC121
C025	C	Conveyor belt	BPP	FCS	II	IIa	C11	ST37	CC37

[a] during production process.
[b] before production process.
[c] food contact surfaces.
[d] non-food contact surfaces.
[e] *L. monocytogenes* strains selected for further characterization.

predominant in 18 Spanish meat processing facilities and meat products, and with ST121, were considered highly adapted to the meat processing environment (Martín et al., 2014). ST121 appears to be expanding in Europe (Bergholz et al., 2018), being detected from foods and food processing environments in Austria, Belgium (Hein et al., 2011), Denmark (Holch et al., 2013) or Switzerland (Stoller et al., 2019). *L. monocytogenes* ST121 strains have specifically the stress survival islet 2 (SSI-2), which is involved in pathogen survival under alkaline and oxidative stress caused by exposure to some commonly used disinfectants in the food industry. This fact suggested that SSI-2 is part of a diverse set of molecular determinants contributing to niche specific adaptation and persistence of *L. monocytogenes* ST121 strains (Harter et al., 2017). ST87 was also detected by Martín et al. (2014) in the 6.6% of FCS of Spanish meat processing plants and meat products, and it was

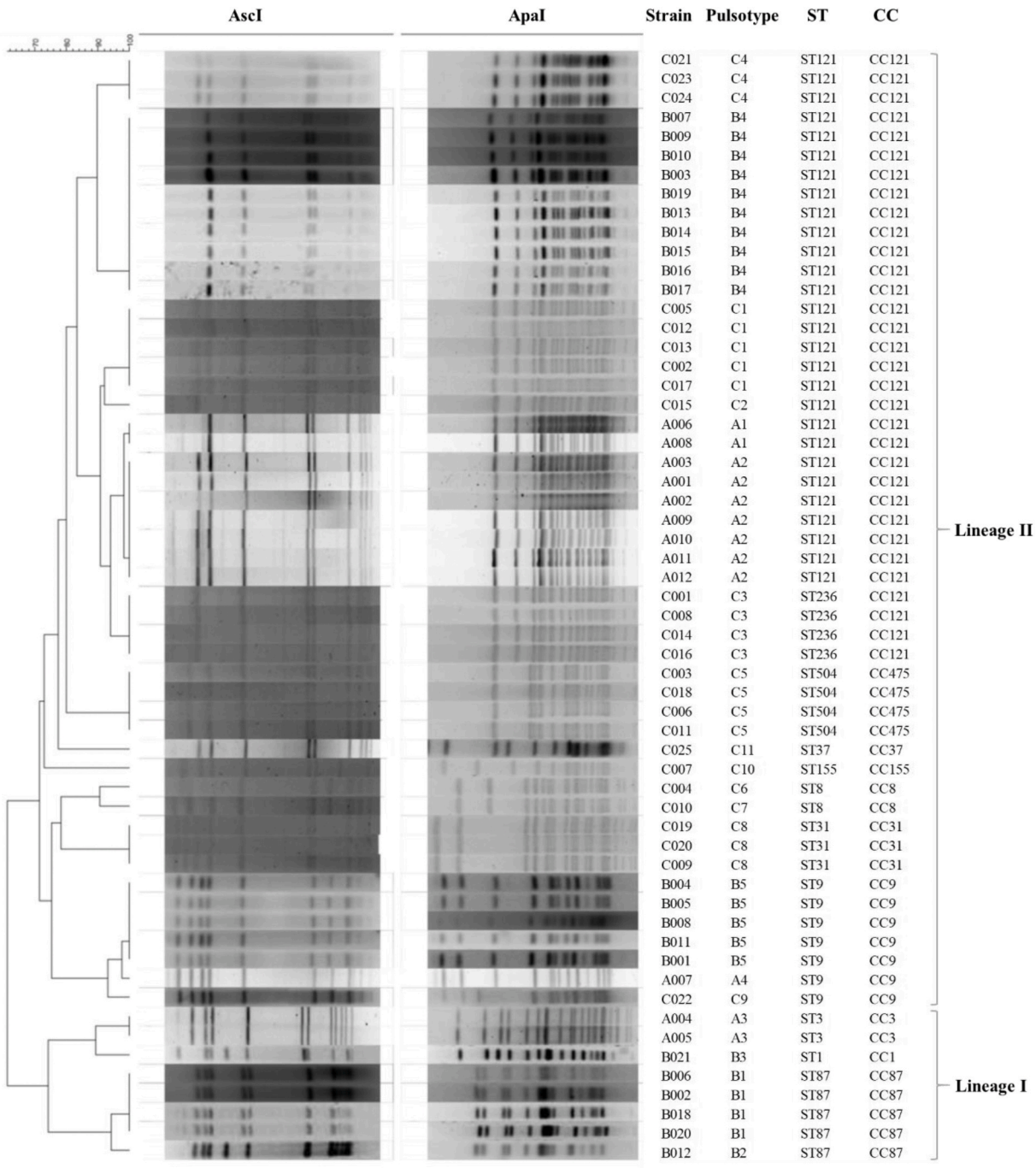

Fig. 1. UPGMA dendrogram showing similarities among AscI and ApaI restriction profiles of *L. monocytogenes* isolates from the deboning and slicing areas of three dry-cured ham processing facilities.

the third most abundant ST (9.2%) found in food samples from different Chinese provinces (Wang et al., 2018).

Pathogenic potential among *L. monocytogenes* isolates is highly heterogeneous (Maury et al., 2016; Orsi et al., 2011). CC1, CC2, CC4, and CC6 have been identified as hypervirulent (Maury et al., 2016) whereas CC9 and CC121, associated to a food origin, are considered hypovirulent (Maury et al., 2019). CC87 is common among food sources, but it has also been linked to human infections (Li et al., 2020; Pérez-Trallero et al., 2014; Zhang et al., 2019). CC87 found in this work was detected only in one sampling carried out in plant B and was not repeatedly isolated over time, which could be explained by the reported absence of stress islands in strains ascribed to this CC (Li et al., 2020). The strain IVb found in this work was ascribed to CC1 (ST1), isolated once from a drain during processing, but not found thereafter. CC1 strains have been associated with more severe listeriosis linked to *Listeria* pathogenicity island 4 (LIPI-4), which increases competitiveness in the gastrointestinal tract (Maury et al., 2016). Further, CC1 strains harboured the *Listeria* genomic island 2 (LGI2), which mediates arsenic resistance related to virulence (Lee et al., 2017). CC1 strains were frequently associated with bacteremia and central nervous system and materno-neonatal infections, while CC121 and CC9 strains appear very infrequently (Maury et al., 2016), just like other CCs found in this study, such as CC8, CC37 and CC155.

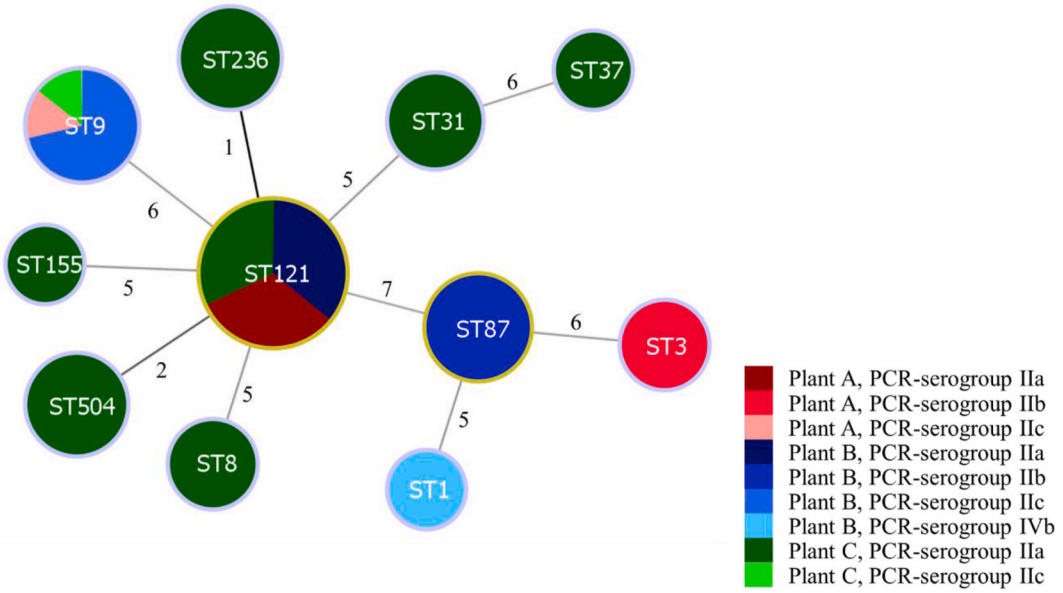

Fig. 2. Minimum spanning tree (MST) of 58 *L. monocytogenes* isolates from deboning and slicing areas of three dry-cured ham processing facilities based on MLST data. Each circle corresponds to one ST with its number indicated inside. The size of each group is proportional to the number of strains. The connection lines between circles differ based on the number of allelic differences between the corresponding STs.

3.3. Virulence and persistence of *L. monocytogenes* strains

Twelve *L. monocytogenes* strains representing the three clonal complexes more frequent in these dry-cured ham processing plants (CC121, CC9 and CC87) were selected for further analysis (Table 4). Strain B021 (ST1, CC1) was also chosen as CC1 was considered hypervirulent and the most commonly associated to human listeriosis (Maury et al., 2016).

3.3.1. Genetic analysis of virulence

Two virulence-associated genes (*inlA* and a *prfA* fragment) were sequenced and analysed to characterize possible virulence differences among the selected strains. Amino acid polymorphisms in InlA sequences are shown in Fig. 3. Two InlA premature stop codon (PMSC) mutations were found in 7 of the 12 strains tested (Table 4), all ascribed to lineage II. *L. monocytogenes* strains A001, A004, B007, C002, C015 and C021, belonging to ST121 (CC121), carried PMSC6 (amino acid 492, nucleotide 1474). *L. monocytogenes* strain B001 ST9 (CC9) possessed PMSC19 (amino acid 326, nucleotide 976), while the other two ST9 strains A007 and C022 showed an InlA sequence identical to that of *L. monocytogenes* EGDe. *L. monocytogenes* B002 and B012 (ST87, CC87) and B021 (ST1, CC1) presented non-synonymous mutations along the sequence with amino acid sequence changes which did not result in PMSC. For the *prfA*, single-nucleotide polymorphisms were observed over the entire sequence of these fragments, but these mutations did not cause an amino acidic change, so truncated PrfA have not been found in this work (Table 4).

Table 4
Genetic analysis of virulence (*inlA* and *prfA*) and resistance to disinfectants (*qacH/radC* and *bcrABC*) of 12 *L. monocytogenes* strains.

Strain	Sequence Type	Virulence		Resistance to disinfectants		
		inlA[a]	*prfA*	*radC*	*qacH*	*bcrABC*
A001	ST121	PMSC6	nd	+[c]	+	–
A007	ST9	nd[b]	nd	–[d]	–	–
A009	ST121	PMSC6	nd	–	–	–
B001	ST9	PMSC19	nd	–	–	–
B002	ST87	nd	nd	–	–	–
B007	ST121	PMSC6	nd	–	–	–
B012	ST87	nd	nd	–	–	–
B021	ST1	nd	nd	–	–	–
C002	ST121	PMSC6	nd	+	+	–
C015	ST121	PMSC6	nd	–	–	–
C021	ST121	PMSC6	nd	+	+	–
C022	ST9	nd	nd	–	–	–

[a] premature stop codons (PMSCs) identified in the *inlA* gene.
[b] PMSCs not detected.
[c] gene detected by PCR.
[d] gene not detected by PCR.

The virulence factor internalin A (InlA), encoded by the *inlA* gene, plays a critical role in crossing the intestinal barrier during the initial stages of an infection (Lecuit et al., 2001). Almost all isolates causing listeriosis in humans express a full-length functional InlA (Ragon et al., 2008). However, a significant proportion of *L. monocytogenes* from RTE foods possess mutations leading to a PMSC in the *inlA* (Chen et al., 2011) that produces a truncated or non-secreted InlA resulting in virulence attenuated phenotypes. Twenty-one naturally occurring mutations causing PMSCs in the *inlA* have been identified (Gelbíčová et al., 2015, 2016; Van Stelten et al., 2010), mainly related with lineage II strains. Two different PMSCs were identified in this work. PMSC6, described by Olier et al. (2003), was detected in all tested ST121 (CC121) strains. This PMSC was also detected by Ragon et al. (2008) in ST121 strains and by Ortiz et al. (2016) in *L. monocytogenes* serotype 1/2a strains. The other PMSC found, PMSC19, was first described by Gelbíčová et al. (2015) in a *L. monocytogenes* serotype 1/2c strain isolated from neonatal listeriosis. PMSC19 was present in one ST9 (CC9) strain (B001) from plant B but not in the other two ST9 strains tested, which implies that could be a strain-specific mutation.

PrfA protein, encoded by the *prfA* gene, is a key regulatory factor for the differential expression of virulence genes of *L. monocytogenes* (Sheehan et al., 1995). Polymorphisms in the *prfA* sequence have been described and related to an attenuation of virulence, giving rise to truncated proteins due to a PMSC, or affecting domains involved in PrfA activity (Velge et al., 2007). In the present study, the mutations observed in the *prfA* sequences have not resulted in changes in the open reading frame nor have generated PMSCs, and therefore, no truncated *prfA* sequences have been detected. The incidence of truncated *prfA* sequences was also very low among the PTs characterized in meat processing plants (López-Alonso et al., 2020; Palma et al., 2017).

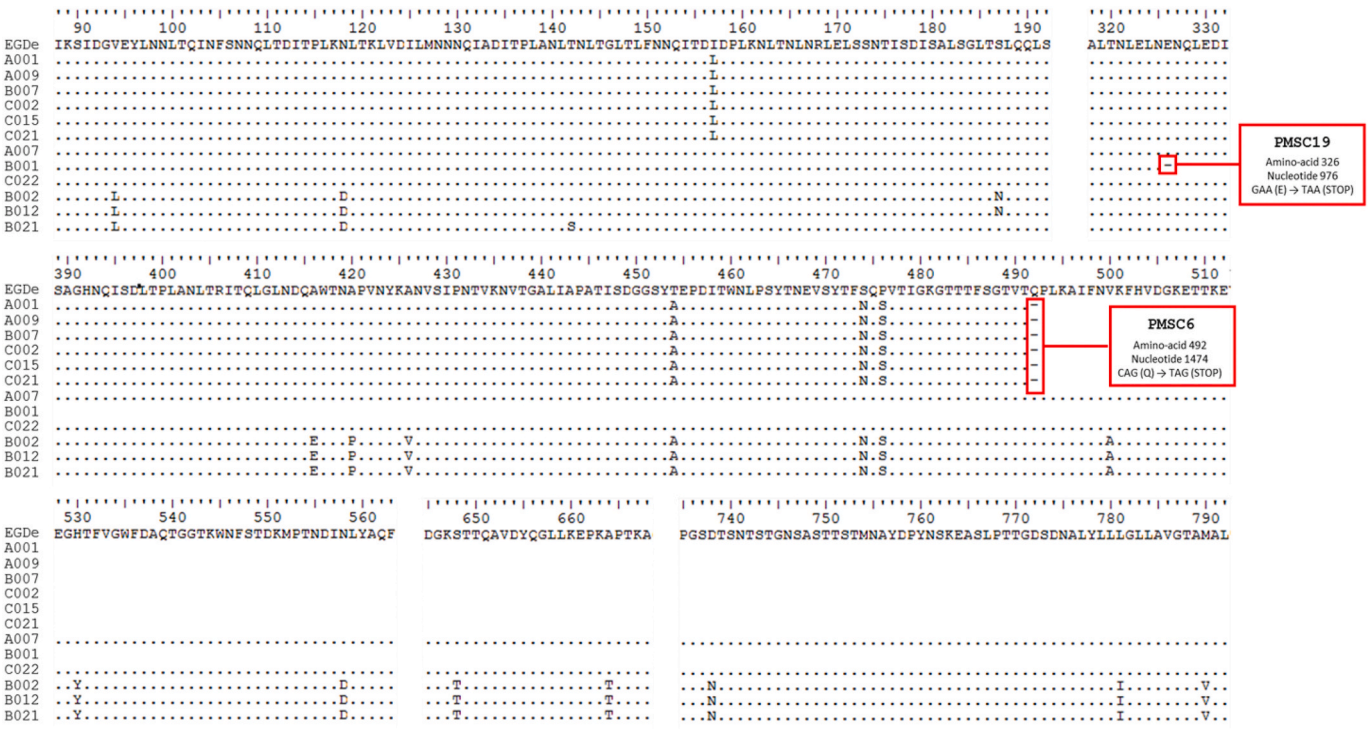

Fig. 3. Amino acid polymorphisms (or non-synonymous mutations) in InlA of 12 *L. monocytogenes* strains belonging to different CCs (CC121: A001, A009, B007, C002, C015 and C021; CC9: A007, B001 and C022; CC87: B002 and B012; and CC1: B021). Amino acid sequences are aligned to the reference strain EGDe. Identical amino acids to the sequence in reference strain are presented as dots (.). Non-sense mutations are indicated by a hyphen (–).

3.3.2. Detection of genetic markers of resistance to disinfectants

The presence of the *qacH, radC* and *bcrABC,* in the selected *L. monocytogenes* strains are summarized in Table 4. Our results indicate that only three of the strains, *L. monocytogenes* A001, C002 and C021, ascribed to ST121 and CC121, have integrated the transposon Tn*6188* as the PCR results were positive for the *qacH* and the *radC* gene size changed from 200 to 5310 bp. The *bcrABC* cassette was not found in any of the tested strains.

Tolerance to quaternary ammonium compounds (QACs) such as the widely used benzalkonium chloride (BC) is especially relevant to *Listeria* adaptations in food-related environments. This tolerance can be due to mutations caused by sublethal BC levels, which enable *a priori* sensitive strains to become resistant (Elhanafi et al., 2010). Further, sublethal concentrations of BC increase *L. monocytogenes* tolerance to some antibiotics and toxic compounds (Xu et al., 2016). Naturally resistant strains to BC by plasmid acquisition have been reported (Kropac et al., 2019). A transposon-based molecular mechanism for BC resistance (Tn*6188*) has been identified and characterized (Müller et al., 2013). This transposon, which contains a small multidrug resistance protein (QacH) associated with the resistance to BC and other QACs (Müller et al., 2013, 2014), is integrated chromosomally within the *radC L. monocytogenes* gene. In our work, three out of 12 strains tested (25.0%), all of them ascribed to CC121, have incorporated the transposon Tn*6188*, which could contribute to the persistence of this clone. Accordingly, Müller et al. (2013) detected 10 out of 91 *L. monocytogenes* strains (11.0%) with different serovars harbouring the Tn*6188*. This transposon was only found in one strain out of 29 isolated by Ortiz et al. (2010).

Elhanafi et al. (2010) identified a BC resistance cassette *bcrABC* in *L. monocytogenes* strains, whose transcription is induced in presence of BC. In this work, the *bcrABC* cassette was not found in any of the tested strains. Similar results were obtained by Ortiz et al. (2016). By contrast, most of 116 BC-resistant strains of diverse serotypes and sources (clinical, environment of food processing plants, and food) had these resistance genes (Dutta et al., 2013), which could suggest that tolerance to BC is often due to the presence of this genetic determinant.

3.3.3. Biofilm formation

Biofilm production by *L. monocytogenes* strains was evaluated during 4 d at ambient temperature (22 °C) and during 7 d at a low temperature (8 °C) mimicking conditions in the dry-cured ham processing plants. None of the isolates were able to form biofilms at 8 °C for 7 d, while all strains showed ability to form biofilm at 22 °C for 4 d, with the exception of *L. monocytogenes* C015 (Fig. 4). The majority of ST121 (CC121) strains and one of the ST9 (CC9) (B001) showed higher biofilm formation under these conditions. *L. monocytogenes* A001 (CC121), isolated from the surface of a ham bone saw, was considered the highest biofilm producer at 22 °C for 4 d, while C015 (CC121), obtained from the surface of a press, exhibited the lowest capacity.

The presence of persistent *L. monocytogenes* strains in food processing environment has been linked to biofilm formation (Manso et al., 2019; Martínez-Suárez et al., 2016). However, high ability to adhere to surface or to produce sessile biomass may not be a prerequisite to persistence or prevalence of *L. monocytogenes* in the food industry (Lee et al., 2019). According to Borucki et al. (2003), strains belonging to lineage II are generally more capable of biofilm formation compared to strains of lineage I, and more abundant in food processing installations, as observed in this work, where lineage II strains (CC121 and CC9) were better biofilm producers. This fact, together with the presence of the transposon Tn*6188*, could contribute to the persistence of CC121 in the deboning areas of dry-cured ham processing plants.

Differences among isolates from the same CC were found in this study, as strains from CC121 were low or high biofilm formers. These intra-genotype variations observed in some CCs, suggest that minor genetic variants within a genotype may influence biofilm formation. Differences in biofilm formation were recorded at the two temperatures studied. According to Stoller et al. (2019), double the biofilm mass was recovered after 96 h of growth at 22 °C compared to 168 h growth at 8 °C, and biofilm production was more than five time greater at 37 °C than at 10 °C after 24 h of incubation (Lee et al., 2019). In this work, all strains, with the exception of C015, were able to form biofilm at 22 °C for 4 d, while reproduction of *L. monocytogenes* biofilm at low

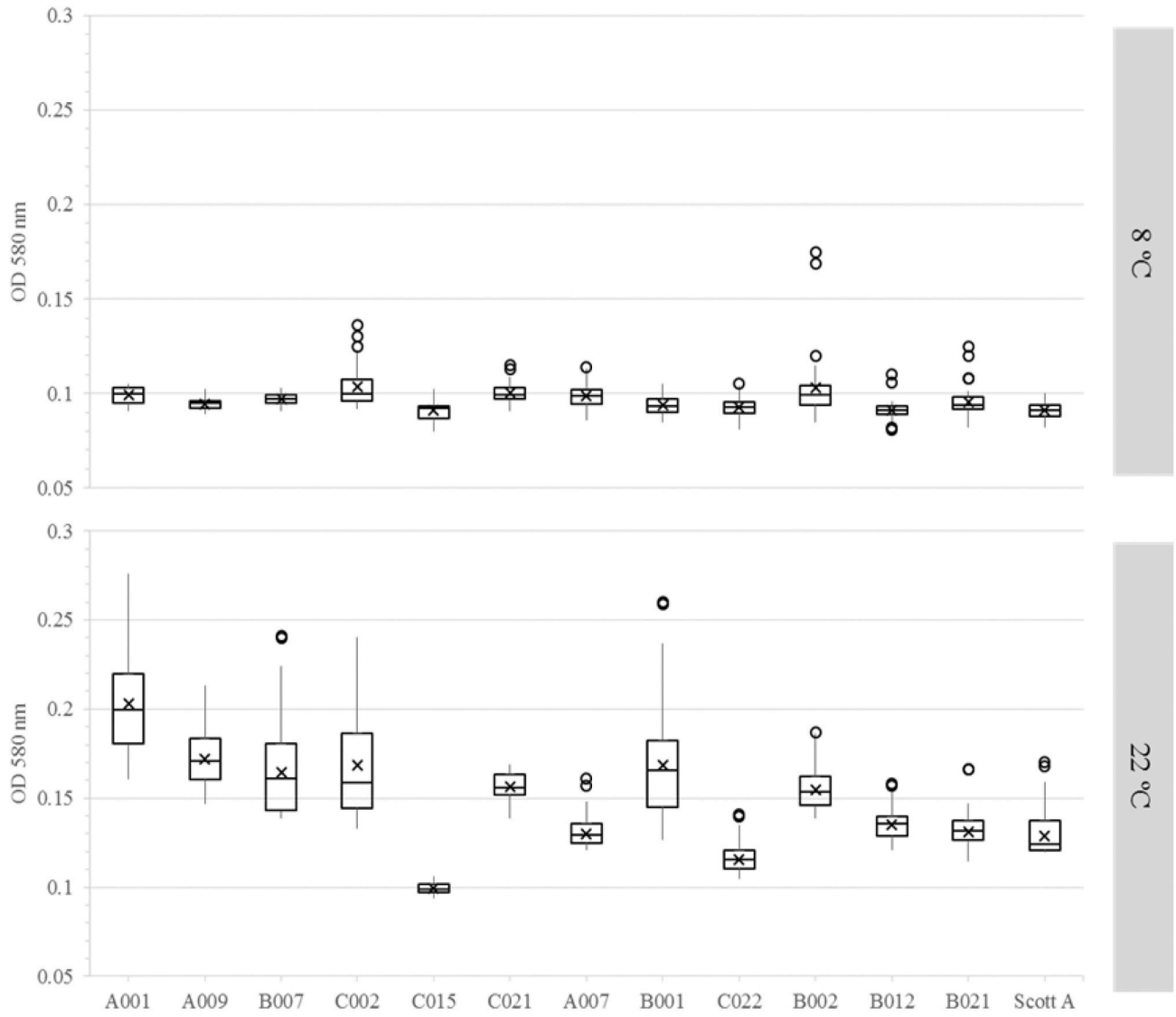

Fig. 4. Biofilm formation by 12 *L. monocytogenes* strains belonging to different CCs (CC121: A001, A009, B007, C002, C015 and C021; CC9: A007, B001 and C022; CC87: B002 and B012; and CC1: B021) and Scott A strain at 8 and 22 °C. The results are presented as box plots of absorbance (OD_{580}) of crystal violet from distained biofilms formed by *L. monocytogenes* strains at each temperature (n = 6). Whiskers extend to minimum and maximum values, and the horizontal line and the symbol (x) in the box represent the median and mean values, respectively.

temperatures under laboratory conditions was not achieved. However, *L. monocytogenes* is capable of forming biofilms in food processing environments (Lee et al., 2019), where temperatures are around 8–10 °C, implying that this biofilm formation capability occurs only under specific environmental conditions. The presence of salt and sublethal concentrations of disinfectants, frequently found in dry-cured ham processing facilities, could contribute to improve the efficacy in biofilm formation by some strains of *L. monocytogenes* (Lee et al., 2019; Maury et al., 2019; Pan et al., 2010).

4. Conclusions

Our study provides novel information on the characterization of *L. monocytogenes* strains from the deboning areas of three dry-cured ham processing facilities and the presence of persistent strains in food contact surfaces after cleaning and disinfection procedures. *L. monocytogenes* CC121 and CC9, two lineage II clones considered hypovirulent and highly adapted to the meat processing environment, were the most abundant in all the facilities investigated. CC121 strains were characterized as strong biofilm formers and some of them harboured the Tn*6188* transposon, responsible for tolerance to QACs. These facts could contribute to the persistence of *L. monocytogenes* CC121 in the dry-cured ham processing environment. However, CC121 strains produced a truncated InlA resulting in virulence attenuated phenotypes. The detection of persistent strains of *L. monocytogenes* in the deboning areas of dry-cured ham processing plants highlights the need to improve the control measures and cleaning and disinfection procedures to reduce the prevalence of this pathogen. The clonal structure of the persistent strains and their virulence determinants should be also considered for the exhaustive elimination of *L. monocytogenes* in dry-cured ham processing environment.

Declaration of competing interest

The authors declare that there is not conflict of interest regarding the publication “Genomic diversity and characterization of *Listeria monocytogenes* from dry-cured ham processing plants” by Aida Pérez-Baltar et al.

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Capítulo 2

Aplicación de tratamientos de inactivación de *L. monocytogenes* en jamón curado

El jamón curado es un producto cárnico RTE que se considera seguro desde el punto de vista microbiológico. Sin embargo, la presencia de *L. monocytogenes* en las plantas de procesado hace que este producto pueda contaminarse durante las operaciones de postprocesado, como el deshuesado y el loncheado, comprometiendo la seguridad microbiológica del mismo o simplemente alcanzando niveles del patógeno que dificultarían su exportación a países con tolerancia cero como EE. UU. La industria del jamón curado español demanda soluciones para ofrecer productos seguros y de calidad, sin que resulten afectadas sus características sensoriales. Por tanto, el segundo objetivo de esta tesis fue ensayar tratamientos de inactivación frente a *L. monocytogenes* en jamón curado. En la primera parte del capítulo, se investigó por primera vez el efecto antimicrobiano de las APH frente a *L. monocytogenes* en jamones deshuesados enteros. En la segunda parte, se evaluó el efecto de tratamientos combinados de APH y bacteriocinas o aceites esenciales frente al patógeno en jamón curado loncheado. Los resultados obtenidos se recogen en dos publicaciones científicas incluidas en el *Science Citation Index* (SCI):

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Listeria monocytogenes inactivation in deboned dry-cured hams by high pressure processing

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ABSTRACT

The effect of high pressure processing (HPP) on *Listeria monocytogenes* inactivation on the surface and in the interior of deboned dry-cured ham was investigated. Whole deboned hams were pressurized at 450 MPa for 10 min or 600 MPa for 5 min and stored for 60 d at 4 and 12 °C. In control non-pressurized dry-cured hams, *L. monocytogenes* counts decreased on the surface throughout refrigerated storage, whereas remained unchanged in the interior of the product. Treatments at 600 MPa reduced *L. monocytogenes* population by 2 logs on the surface and by 3 logs in the interior of hams. Minor changes in the physicochemical characteristics were found. High water activity in the interior of dry-cured ham might enhance the pathogen inhibition by HPP. Treatment at 600 MPa for 5 min in whole dry-cured ham allowed to reach the Food Safety Objective for *L. monocytogenes* according to the European and the USDA criteria in case of contamination during deboning.

1. Introduction

Listeria monocytogenes causes listeriosis, an atypical and severe foodborne disease that affects especially susceptible groups such as pregnant women, newborn infants, children, elderly and immunocompromised individuals. Listeriosis incidence is increasing in Europe in the last years, with a fatality rate of 13.8% in 2017 (EFSA, 2018). *L. monocytogenes* is able to grow under different stress conditions, such as refrigeration temperatures, high salt concentration and reduced water activity (Lado & Yousef, 2007), and can form biofilms on the surface of equipments, pipes and drains of the food-processing environments (Colagiorgi et al., 2017). Foods are a major vehicle for listeriosis, particularly ready-to-eat (RTE) foods that have been exposed to the processing environment (Buchanan, Gorris, Hayman, Jackson, & Whiting, 2017).

Dry-cured ham is considered as a microbiologically safe product due to its low water activity and high salt content. Nevertheless, it can be contaminated with foodborne pathogens during the industrial processing (e.g. deboning) or as a result of poor hygiene practices. Food safety regulations in the European Union established a maximum of 100 cfu/g of *L. monocytogenes* for RTE products during their shelf-life (European Commission, 2005), while a "zero tolerance" criterion is required in the USA (USDA-FSIS, 2014). The control of *L. monocytogenes* in dry-cured sliced ham by biopreservation and/or intervention technologies (e.g. high hydrostatic pressure treatments) to obtain safer products would boost the international trade of this traditional food product.

The industrial implementation of high pressure processing (HPP) is constantly increasing due to its efficiency to control foodborne pathogens and spoilage microorganisms. HPP has been recommended by regulatory agencies (i.e. Food Safety and Inspection Service (FSIS) or Health Canada) as an effective post-packaging technology to control *L. monocytogenes* mainly in RTE meats (Health Canada, 2014; USDA-FSIS, 2012). Moreover, such efficacy has been demonstrated in different meat products, including cooked ham (Hereu, Dalgaard, Garriga, Aymerich, & Bover-Cid, 2012; Montiel, Martín-Cabrejas, & Medina, 2015) or sliced dry-cured ham (De Alba, Bravo, & Medina, 2015; Hereu, Bover-Cid, Garriga, & Aymerich, 2012), but not, to our knowledge, in whole pieces of deboned dry-cured hams. Therefore, the purpose of this work was to evaluate the effect of HPP (450 MPa for 10 min or 600 MPa for 5 min) on the inactivation of *L. monocytogenes* on the surface and in the interior of whole deboned dry-cured ham stored at 4 and 12 °C for 60 days. The effect on the physicochemical characteristics of ham was also investigated.

2. Materials and methods

2.1. Microorganisms

L. monocytogenes S2 (serotype 1/2a), S4–2 (serotype 1/2b), S7–2 (serotype 4b) and S12-1 (serotype 1/2c), isolated from an Iberian pig

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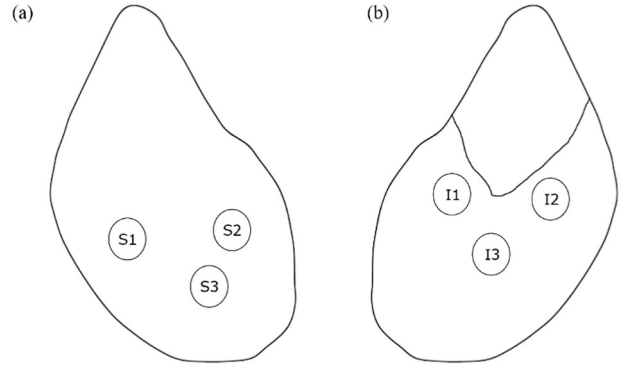

Fig. 1. Inoculation areas at the muscular (a) and the fatty side (b) of the surface (S1, S2 and S3) and the interior (I1, I2 and I3) of deboned dry-cured hams.

processing plant and previously characterized by Ortiz et al. (2010), were used as test organisms. The strains were maintained at −80 °C in Brain Heart Infusion broth (BHI, Biolife S.r.l., Milan, Italy) with 20% glycerol. A four-strain cocktail of *L. monocytogenes* was prepared by mixing equal amounts of cultures of *L. monocytogenes* strains, separately grown in BHI for 18 h at 37 °C.

2.2. Inoculation of deboned dry-cured hams

Forty-five whole deboned dry-cured hams were obtained from a commercial supplier in Spain. Hams were marked with a branding iron and red ink in 6 different circular areas, 5 cm in diameter, 3 at the muscular side (S1, S2, and S3) and another 3 at the fatty side (I1, I2 and I3) in order to contaminate the surface and the interior of dry-cured hams, respectively (Fig. 1). Surface and interior were inoculated to achieve a final population of approximately 10^6 cfu per inoculated zone, using the *L. monocytogenes* cocktail. Inoculated hams were held at room temperature for 2 h to allow cell attachment before vacuum-packaging.

2.3. Pressurization of samples

Deboned dry-cured hams were individually vacuum-packaged in high-barrier multilayer bags (365 mm × 550 mm and 160 μm thickness, Tofilm S.L., Murcia, Spain) and divided into three groups: control (non-pressurized), 450/10 (pressurized at 450 MPa for 10 min) and 600/5 (pressurized at 600 MPa for 5 min). Hams were held at 4 °C until pressurization to allow pathogen adaptation to cold temperatures. High pressure processing was carried out in a 135-l-capacity isostatic press (NC, Hiperbaric, Burgos, Spain). The compression rate was approximately 215 MPa/min whereas the pressure release time was 6 s. The water temperature used as pressure-transmitting medium was 18 °C. After treatments, pressurized and non-pressurized deboned dry-cured hams were stored under strict refrigeration temperatures (4 °C) and temperature abuse conditions (12 °C) for 60 d. Three independent trials were carried out.

2.4. Sample collection and microbiological analysis

To provide an adequate recovery of the target microorganism, inoculated areas from the surface of dry-cured hams (5 cm in diameter and approximately 6.0–8.0 mm thick) were removed with a sterile knife. Meat cylinders (2.8 cm in diameter and 3 cm length) were taken from each internal inoculation point using a sterile borer. *L. monocytogenes* counts were determined at 1, 30 and 60 d after pressurization. Samples were diluted with 100 ml sterile 0.1% (wt/vol) peptone water solution in stomacher bags and homogenized twice for 120 s using a Silver Masticator homogenizer (IUL Instruments, Barcelona, Spain). Decimal dilutions of the homogenates were prepared in the same sterile solution and were spread on duplicate plates of CHROMagar *Listeria* (CH-L, Scharlab S.l., Barcelona, Spain) incubated at 37 °C for 48 h.

2.5. Physicochemical analysis

For the analysis of physicochemical characteristics, non-inoculated samples were taken from the surface and the interior of dry-cured hams and minced with a meat chopper to determine pH, water activity (a_w), moisture content (MC), sodium chloride (NaCl) and nitrites (NO^{2-}) content. pH was determined with a pH-meter GLP22 (Crison Instruments S.A., Barcelona, Spain) in samples (5 g) homogenized with 15 ml of distilled water. Water activity was measured in 5 g dry-cured ham samples using an Aqualab Series 3 water activity meter (Decagon Devices Inc., Washington, USA). Moisture content was determined according to ISO 1442 (1997). Nitrites content was analysed using the reference method from ISO 2918 (1975). Salt content was estimated as chloride using QUANTAB Chloride Titrator according to the Association of Official Analytical Chemists (AOAC, 2012). All physicochemical determinations were performed in triplicate samples at 1, 30 and 60 d after pressurization.

2.6. Statistical analysis

Analysis of variance (ANOVA) with treatment, zone of ham, time and temperature of storage as main effects and their interactions was carried out using SPSS Statistics 22.0 software (IBM Corp., Armonk, NY, USA). Significant differences between means were assessed by Tukey's test for a confidence interval of 95% (Steel, Torrie, & Dickey, 1996).

3. Results and discussion

3.1. Listeria monocytogenes

L. monocytogenes counts in control and treated deboned dry-cured hams during refrigerated storage at 4 and 12 °C are listed in Table 1. Significant effect ($P < .001$) of treatment, zone of ham, time and temperature of storage on *L. monocytogenes* counts was shown by ANOVA. One day after treatments, *L. monocytogenes* counts in control samples were 5.1 and 6.1 log cfu on the surface and in the interior of hams, respectively. Reductions of 0.9 and 1.4 log units were found on the surface and in the interior, respectively, when 450 MPa were applied for 10 min. Reductions of 1.9 and 2.9 log units in pathogen population on the surface and in the interior of hams, respectively, were

Table 1
Listeria monocytogenes counts (log cfu per inoculated zone) in deboned dry-cured ham treated with HPP at 450 MPa for 10 min (450/10) or with 600 MPa for 5 min (600/5), stored for 60 d at 4 and 12 °C.

			1 day	30 days	60 days
4 °C	Surface	NP	5.1 ± 0.4^{bB}	4.6 ± 0.3^{bAB}	3.8 ± 0.3^{bA}
		450/10	4.2 ± 0.4^{abB}	2.5 ± 0.3^{aA}	2.6 ± 0.4^{abA}
		600/5	3.2 ± 0.4^{aB}	1.8 ± 0.3^{aA}	1.7 ± 0.3^{aA}
	Interior	NP	6.1 ± 0.2^{cA}	6.1 ± 0.3^{cA}	6.2 ± 0.3^{cA}
		450/10	4.7 ± 0.2^{bA}	4.4 ± 0.3^{bA}	4.2 ± 0.3^{bA}
		600/5	3.2 ± 0.2^{aA}	2.5 ± 0.2^{aA}	2.7 ± 0.2^{aA}
12 °C	Surface	NP	5.1 ± 0.4^{bC}	3.7 ± 0.3^{bB}	2.3 ± 0.3^{aA}
		450/10	4.2 ± 0.4^{abB}	2.5 ± 0.3^{aA}	1.6 ± 0.3^{aA}
		600/5	3.2 ± 0.4^{aB}	1.6 ± 0.3^{aA}	1.5 ± 0.3^{aA}
	Interior	NP	6.1 ± 0.2^{cA}	5.9 ± 0.2^{cA}	5.9 ± 0.2^{cA}
		450/10	4.7 ± 0.2^{bA}	4.3 ± 0.2^{bA}	4.2 ± 0.2^{bA}
		600/5	3.2 ± 0.2^{aB}	2.4 ± 0.2^{aA}	2.5 ± 0.2^{aA}

NP, non-pressurized.
Values are means ± SE.
[a, b, c]Means within the same column with different superscripts differ significantly at $P < .05$ for a given zone of dry-cured ham and temperature.
[A, B, C]Means within the same row with different superscripts differ significantly at $P < .05$.

achieved after HPP at 600 MPa for 5 min.

During the 60 d of refrigerated storage, *L. monocytogenes* decreased on the surface of non-pressurized hams, whereas the pathogen levels remained practically constant in the interior at both temperatures. The population of *L. monocytogenes* on the surface of 450 MPa treated hams was 1.2 and 0.7 log units lower than in non-pressurized hams, respectively, after 60 d at 4 and 12 °C. In the interior of pressurized hams, pathogen counts were 2.0 and 1.7 log units lower. Surface samples treated at 600 MPa showed counts 2.1 and 0.8 log units lower than control samples at 4 and 12 °C, respectively. This treatment reduced bacteria levels in the interior of hams by around 3.4 log units with respect to the control after 60 d of storage at both temperatures.

L. monocytogenes population decreased by < 1 log unit on the surface of whole deboned dry-cured ham treated at 450 MPa for 10 min. This result was in agreement with those reported in sliced dry-cured ham pressurized under the same conditions (De Alba et al., 2015; Pérez-Baltar, Serrano, Bravo, Montiel, & Medina, 2019). However, higher reductions of the pathogen were found in the interior of deboned hams pressurized at 450 and 600 MPa. Differences in the behaviour of *L. monocytogenes* between the surface and the interior of whole hams could be explained by differences in water activity. The higher a_w in the interior might have contributed to increase the inactivation of the pathogen. As previously observed by Hereu, Bover-Cid, et al. (2012), HPP at 600 MPa for 5 min achieved a range of 2–3 log units for pathogen reduction. In this work, 600 MPa for 5 min reached reductions of 2.1 log cfu on the surface of dry-cured hams after 60 days of storage at 4 °C. This pressure treatment in the interior of cured ham attained reductions around 3.4 log units, both at 4 and 12 °C, achieving the European Food Safety Objective (FSO) as well as the Performance Criterion (PC) of 2.4 log reductions suggested as a listericidal treatment for dry-cured ham (Hoz, Cambero, Cabeza, Herrero, & Ordóñez, 2008). According to our results, *L. monocytogenes* declined more rapidly on the surface of deboned dry-cured hams than in the interior of the product. Differences in the physicochemical characteristics might affect the behaviour of the pathogen.

3.2. Physicochemical analysis

Physicochemical properties of whole deboned dry-cured ham pressurized at 450 MPa for 10 min or 600 MPa for 5 min for 60 d at 4 °C and 12 °C are listed in Tables 2–6. Values of pH (Table 2) ranged from 5.42 to 6.03 in control and pressurized samples at 4 °C, and from 5.67 to 6.03 at 12 °C. A slight decrease of pH values was registered in pressurized samples from the interior of ham 1 day after treatment, but these values tended to stabilize and no differences were recorded among control and pressurized dry-cured hams after 60 d of storage. De Alba, Bravo, and Medina (2013) observed a minimal pH increase in pressurized dry-cured ham at 400–500 MPa and these differences also tended to attenuate during refrigerated storage. Nevertheless, no statistical differences ($P > .05$) in pH values were detected in the present work when pressure was applied neither on the surface nor in the interior of hams. Although pH is an important barrier for pathogen growth, acidity is not a significant hurdle in dry-cured ham (Stollewerk, Jofré, Comaposada, Arnau, & Garriga, 2012).

Table 2
Values of pH in deboned dry-cured ham treated with HPP at 450 MPa for 10 min (450/10) or with 600 MPa for 5 min (600/5), stored for 60 d at 4 and 12 °C.

			1 day	30 days	60 days
4 °C	Surface	NP	5.87 ± 0.06^{abA}	5.74 ± 0.03^{aA}	5.81 ± 0.06^{aA}
		450/10	5.95 ± 0.06^{bB}	5.64 ± 0.03^{aA}	5.81 ± 0.06^{aAB}
		600/5	5.70 ± 0.06^{aA}	5.86 ± 0.03^{bB}	5.95 ± 0.06^{aB}
	Interior	NP	6.03 ± 0.03^{bA}	5.88 ± 0.03^{abA}	5.93 ± 0.39^{aA}
		450/10	5.90 ± 0.03^{aA}	5.83 ± 0.03^{aA}	5.90 ± 0.39^{aA}
		600/5	5.92 ± 0.03^{aA}	5.97 ± 0.03^{bA}	5.42 ± 0.39^{aA}
12 °C	Surface	NP	5.87 ± 0.06^{abB}	5.67 ± 0.03^{aA}	5.75 ± 0.05^{aAB}
		450/10	5.95 ± 0.06^{bA}	5.86 ± 0.03^{bA}	5.91 ± 0.05^{aA}
		600/5	5.70 ± 0.06^{aA}	5.73 ± 0.03^{aA}	5.73 ± 0.05^{aA}
	Interior	NP	6.03 ± 0.03^{bC}	5.72 ± 0.04^{aA}	5.87 ± 0.03^{aB}
		450/10	5.90 ± 0.03^{aA}	6.00 ± 0.04^{bB}	5.96 ± 0.03^{aAB}
		600/5	5.92 ± 0.03^{aB}	5.79 ± 0.04^{aA}	5.86 ± 0.03^{aAB}

NP, non-pressurized.
Values are means ± SE.
[a, b]Means within the same column with different superscripts differ significantly at $P < .05$ for a given zone of dry-cured ham and temperature.
[A, B, C]Means within the same row with different superscripts differ significantly at $P < .05$.

Significant ($P < .001$) effects of treatment, zone of ham and time of storage on dry-cured ham a_w were recorded by ANOVA. Values of a_w in control deboned dry-cured ham on day 1 (Table 3) were 0.864 on the surface and increased to 0.887 and 0.882 after 60 d at 4 and 12 °C, respectively. Lower differences were observed in the interior of the product with values of 0.880 on day 1 and 0.890 on day 60 at both temperatures. One day after treatments, significant differences ($P > .05$) were not found between treated and control samples, whereas minimal differences were found between values obtained with pressurization. These differences were attenuated during storage at 4 and 12 °C on the surface and in the interior of dry-cured hams. In general, values of a_w tended to increase slightly throughout storage on the surface and in the interior, and in control and pressurized samples at the two temperatures assayed. A certain increase of a_w by HPP was previously reported by De Alba, Montiel, Bravo, Gaya, and Medina (2012) in sliced dry-cured ham and by Serra et al. (2007) in whole hams with short ripening times. Low a_w may exert a baroprotective effect on bacteria (Patterson, 2005), and it might contribute to the differences recorded in *L. monocytogenes* inactivation between the surface and the interior of dry-cured ham. Higher resistance of *L. monocytogenes* in inoculated sliced dry-cured ham treated at 600 MPa for 5 min was observed by Hereu, Bover-Cid, et al. (2012) when the a_w was lower. Similarly, Bover-Cid, Belletti, Aymerich, and Garriga (2015) reported reductions of *L. monocytogenes* from 2.2 to 6.8 log cfu/g with the increase of a_w of dry-cured ham from 0.860 to 0.960. This baroprotective effect has been related with the stabilisation of macromolecules such as proteins as a result of the decrease of cell compressibility, caused by the increase of solutes concentration in the cytoplasm of bacteria (Possas, Pérez-Rodríguez, Valero, Rincón, & García-Gimeno, 2018), although the mechanisms have not been clarified.

Moisture content values were significantly ($P < .001$) affected by zone of ham and time of storage. One day after treatment, moisture content (Table 4) was 36.4 and 50.6% on the surface and in the interior of control non-pressurized hams, respectively. These values did not differ significantly ($P > .05$) from those observed in pressurized hams, both on the surface and in the interior. After 60 d at 4 and 12 °C, MC of pressurized samples did not differ from those recorded in control non-pressurized samples, both on the surface and in the interior of hams, with an average value of 46.1%. In the same way, Pietrasik, Gaudette, and Johnston (2017) did not register differences in the MC of cured wieners as a consequence of high pressure. HPP treatments at 600 MPa did not affect the water activity or the moisture content of restructured cured hams (Fulladosa, Serra, Gou, & Arnau, 2009; Picouet et al., 2012) whereas the water holding capacity was reduced.

Nitrite content was influenced by treatment ($P < .05$) and zone of ham ($P < .01$) as indicated by ANOVA. Nitrite concentration (Table 5) at day 1 on the surface and in the interior of control non-pressurized dry-cured hams was 2.2 and 2.7 mg/kg, respectively. Treatments of HPP at 450 and 600 MPa did not significantly ($P > .05$) change nitrite content both on the surface and in the interior of deboned dry-cured hams at 4 and 12 °C. In general, levels were maintained throughout storage on the surface and in the interior, and in control and pressurized samples at the two temperatures assayed. After 60 d, an average value of 2.8 mg/kg was recorded on the surface and in the interior of dry-cured hams. The slight variations found on the surface of pressurized

Table 3
Values of a_w in deboned dry-cured ham treated with HPP at 450 MPa for 10 min (450/10) or with 600 MPa for 5 min (600/5), stored for 60 d at 4 and 12 °C.

			1 day	30 days	60 days
4 °C	Surface	NP	0.864 ± 0.002^{abA}	0.882 ± 0.003^{aB}	0.887 ± 0.004^{aB}
		450/10	0.868 ± 0.002^{bA}	0.882 ± 0.003^{aB}	0.878 ± 0.004^{aB}
		600/5	0.858 ± 0.002^{aA}	0.877 ± 0.003^{aB}	0.882 ± 0.004^{aB}
	Interior	NP	0.880 ± 0.002^{abA}	0.891 ± 0.003^{aB}	0.890 ± 0.003^{aB}
		450/10	0.884 ± 0.002^{bA}	0.892 ± 0.003^{aB}	0.885 ± 0.003^{aAB}
		600/5	0.875 ± 0.002^{aA}	0.884 ± 0.003^{aAB}	0.888 ± 0.003^{aB}
12 °C	Surface	NP	0.864 ± 0.002^{abA}	0.886 ± 0.003^{aB}	0.882 ± 0.002^{aB}
		450/10	0.868 ± 0.002^{bA}	0.885 ± 0.003^{aB}	0.879 ± 0.002^{aB}
		600/5	0.858 ± 0.002^{aA}	0.875 ± 0.003^{aB}	0.886 ± 0.002^{aC}
	Interior	NP	0.880 ± 0.002^{abA}	0.893 ± 0.003^{aB}	0.890 ± 0.002^{aB}
		450/10	0.884 ± 0.002^{bA}	0.894 ± 0.003^{aB}	0.889 ± 0.002^{aAB}
		600/5	0.875 ± 0.002^{aB}	0.883 ± 0.003^{aAB}	0.892 ± 0.002^{aB}

NP, non-pressurized.
Values are means ± SE.
[a, b]Means within the same column with different superscripts differ significantly at $P < .05$ for a given zone of dry-cured ham and temperature.
[A, B, C]Means within the same row with different superscripts differ significantly at $P < .05$.

Table 4
Moisture content (%) in deboned dry-cured ham treated with HPP at 450 MPa for 10 min (450/10) or with 600 MPa for 5 min (600/5), stored for 60 d at 4 and 12 °C.

			1 day	30 days	60 days
4 °C	Surface	NP	36.4 ± 0.6^{abA}	38.0 ± 0.7^{abAB}	40.8 ± 1.3^{aB}
		450/10	38.5 ± 0.6^{bA}	36.0 ± 0.7^{aA}	38.5 ± 1.3^{aA}
		600/5	35.6 ± 0.6^{aA}	40.4 ± 0.7^{bB}	39.6 ± 1.3^{aB}
	Interior	NP	50.6 ± 0.8^{aA}	53.7 ± 0.6^{aB}	54.4 ± 0.9^{aB}
		450/10	50.5 ± 0.8^{aA}	53.2 ± 0.6^{aA}	45.4 ± 0.9^{aA}
		600/5	51.0 ± 0.9^{aA}	53.1 ± 0.6^{aA}	53.2 ± 0.9^{aA}
12 °C	Surface	NP	36.4 ± 0.6^{abA}	40.5 ± 0.8^{bB}	39.2 ± 1.1^{aAB}
		450/10	38.5 ± 0.6^{bA}	37.4 ± 0.8^{aA}	39.7 ± 1.1^{aA}
		600/5	35.6 ± 0.6^{aA}	36.7 ± 0.8^{aA}	40.8 ± 1.1^{aB}
	Interior	NP	50.6 ± 0.8^{aA}	53.8 ± 1.2^{aB}	53.2 ± 0.6^{aAB}
		450/10	50.5 ± 0.8^{aA}	51.6 ± 1.2^{aAB}	54.9 ± 0.6^{aB}
		600/5	51.0 ± 0.9^{aA}	51.3 ± 1.2^{aA}	53.9 ± 0.6^{aB}

NP, non-pressurized.
Values are means ± SE.
[a, b]Means within the same column with different superscripts differ significantly at $P < .05$ for a given zone of dry-cured ham and temperature.
[A, B]Means within the same row with different superscripts differ significantly at $P < .05$.

Table 5
Nitrites content (mg/kg) in deboned dry-cured ham treated with HPP at 450 MPa for 10 min (450/10) or with 600 MPa for 5 min (600/5), stored for 60 d at 4 and 12 °C.

			1 day	30 days	60 days
4 °C	Surface	NP	2.2 ± 0.2^{aA}	2.8 ± 0.2^{aB}	2.3 ± 0.2^{aAB}
		450/10	2.2 ± 0.2^{aA}	2.2 ± 0.2^{aA}	2.8 ± 0.2^{aA}
		600/5	2.5 ± 0.2^{aA}	2.9 ± 0.2^{aA}	2.5 ± 0.2^{aA}
	Interior	NP	2.7 ± 0.3^{aA}	2.4 ± 0.3^{aA}	2.4 ± 0.2^{aA}
		450/10	2.9 ± 0.3^{aA}	3.2 ± 0.3^{aA}	3.2 ± 0.2^{aA}
		600/5	3.0 ± 0.3^{aA}	3.2 ± 0.3^{aA}	2.7 ± 0.2^{aA}
12 °C	Surface	NP	2.2 ± 0.2^{aA}	2.7 ± 0.3^{aA}	2.8 ± 0.2^{aA}
		450/10	2.2 ± 0.2^{aA}	2.5 ± 0.3^{aAB}	3.1 ± 0.2^{aB}
		600/5	2.5 ± 0.2^{aA}	3.3 ± 0.3^{aAB}	3.5 ± 0.2^{aB}
	Interior	NP	2.7 ± 0.3^{aA}	2.7 ± 0.2^{aA}	3.3 ± 0.3^{aA}
		450/10	2.9 ± 0.3^{aA}	2.6 ± 0.2^{aA}	2.5 ± 0.3^{aA}
		600/5	3.0 ± 0.3^{aA}	3.1 ± 0.2^{aA}	2.8 ± 0.3^{aA}

NP, non-pressurized.
Values are means ± SE.
[a, b]Means within the same column with different superscripts differ significantly at $P < .05$ for a given zone of dry-cured ham and temperature.
[A, B]Means within the same row with different superscripts differ significantly at $P < .05$.

Table 6
Sodium chloride content (%, wt/wt) in deboned dry-cured ham treated with HPP at 450 MPa for 10 min (450/10) or with 600 MPa for 5 min (600/5), stored for 60 d at 4 and 12 °C.

			1 day	30 days	60 days
4 °C	Surface	NP	3.1 ± 0.2^{aA}	3.0 ± 0.2^{aA}	3.4 ± 0.2^{aA}
		450/10	3.3 ± 0.2^{aA}	2.9 ± 0.2^{aA}	3.3 ± 0.2^{aA}
		600/5	3.2 ± 0.2^{aA}	3.3 ± 0.2^{aA}	3.3 ± 0.2^{aA}
	Interior	NP	4.1 ± 0.1^{abA}	4.2 ± 0.1^{aA}	4.2 ± 0.2^{aA}
		450/10	4.1 ± 0.1^{aA}	4.0 ± 0.1^{aA}	4.0 ± 0.2^{aA}
		600/5	4.5 ± 0.1^{bA}	4.1 ± 0.1^{aA}	4.6 ± 0.2^{aA}
12 °C	Surface	NP	3.1 ± 0.2^{aA}	2.9 ± 0.1^{bA}	3.0 ± 0.1^{aA}
		450/10	3.3 ± 0.2^{aB}	2.7 ± 0.1^{aA}	3.3 ± 0.1^{aB}
		600/5	3.2 ± 0.2^{aA}	2.8 ± 0.1^{abA}	3.1 ± 0.1^{aA}
	Interior	NP	4.1 ± 0.1^{abA}	3.9 ± 0.1^{aA}	4.2 ± 0.1^{aA}
		450/10	4.1 ± 0.1^{aA}	3.8 ± 0.1^{aA}	4.7 ± 0.1^{bB}
		600/5	4.5 ± 0.1^{bB}	4.2 ± 0.1^{bAB}	4.0 ± 0.1^{aA}

NP, non-pressurized.
Values are means ± SE.
[a, b]Means within the same column with different superscripts differ significantly at $P < .05$ for a given zone of dry-cured ham and temperature.
[A, B]Means within the same row with different superscripts differ significantly at $P < .05$.

hams stored at 12 °C could be attributed to sample-to-sample variation. Sodium nitrite is a key antimicrobial ingredient to control pathogens in RTE meat products, including *L. monocytogenes*. Differences in the pathogen inactivation on the surface of two types of dry-cured hams during aging was partly related with their different nitrite content (Montiel et al., unpublished). Inhibitory activity of nitrite on microbial growth is increased with HPP. According to De Alba, Bravo, Medina, Park, and Mackey (2013), relatively low concentrations of nitrite were sufficient to increase the bactericidal effect of HPP in *L. monocytogenes* cultures. The higher nitrite content registered in the interior of dry-cured hams in the present work could enhance the effectiveness of HPP treatments. In this way, reactive oxygen species (ROS) that appear as a consequence of pressure application (Aertsen, De Spiegeleer, Vanoirbeek, Lavilla, & Michiels, 2005) could react with nitrite radical form (NO^-) and would increase its antimicrobial effect.

NaCl content of deboned hams was significantly influenced by treatment ($P < .05$), zone of ham ($P < .001$) and time of storage ($P < .001$). NaCl content (Table 6) on the surface and in the interior of control hams one day after treatments was 3.1 and 4.1%, respectively. During refrigerated storage, salt content was maintained in non-pressurized hams, whereas minimal variations were found in pressurized samples from the interior of deboned hams stored at 12 °C, which could be attributed to sample-to-sample variation. It seems to be a straight

relationship between NaCl content and the effectiveness of HPP against *L. monocytogenes*. As observed by Morales, Calzada, and Núñez (2006), the lethal effect of high pressure at 450 MPa for 10 min was more pronounced in sliced Iberian than in Serrano ham, which was partly explained by differences in salt concentration. The higher NaCl content in Serrano ham would protect *L. monocytogenes* during pressurization, although the recovery of surviving cells would be impaired under these unfavourable conditions. In the present work, salt in moisture on the surface (8.5%) was slightly higher than in the interior (8.1%) of dry-cured hams, probably affecting pathogen survival after pressurization and during refrigerated storage.

4. Conclusions

L. monocytogenes was inactivated on the surface of whole deboned dry-cured hams during refrigerated storage. On the contrary, *L. monocytogenes* counts remained at constant levels in the interior of hams during the 60 d of storage at 4 and 12 °C. Differences in a_w, salt and nitrite content between the surface and the interior of deboned hams might contribute to the differences recorded in the pathogen inactivation. The application of a pressure treatment at 600 MPa for 5 min would achieve important reductions and the FSO for both European and USDA criterion would be reached in case of contamination in the interior of dry-cured hams during deboning process. This treatment would avoid the recovery of the pathogen under strict refrigeration (4 °C) and temperature abuse conditions (12 °C) for 60 d, without affecting significantly the physicochemical characteristics of dry-cured ham. Thus, based on the results obtained in this study, 600 MPa for 5 min would be recommended to inactivate *L. monocytogenes* in whole deboned dry-cured hams in case of post-processing contamination.

Declaration of Competing Interest

None.

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Prefacio a la Publicación 2.2

Actividad antimicrobiana de aceites esenciales frente a *L. monocytogenes*

Inicialmente, se estudió el efecto antimicrobiano de tres aceites esenciales (AEs) frente a *L. monocytogenes*. Este trabajo, que no ha sido publicado, se planteó con el objetivo de seleccionar el aceite esencial más eficaz para su aplicación en combinación con altas presiones (APH) en jamón curado. Para ello, se determinó la concentración mínima inhibitoria (CMI) de tres AEs frente a un cóctel de cuatro cepas de *L. monocytogenes*. Asimismo, se estudió el efecto inhibitorio de dichos AEs en jamón curado loncheado, inoculado con el cóctel del patógeno, en condiciones de refrigeración (4 ºC) y en condiciones de abuso de temperatura (12 ºC) durante 30 d.

1. Materiales y métodos

1.1. *L. monocytogenes*

Se utilizaron cuatro cepas de *L. monocytogenes* de la colección de microorganismos del INIA, S2 (serotipo 1/2a), S4-2 (serotipo 1/2b), S7-2 (serotipo 4b) y S12-1 (serotipo 1/2c), aisladas previamente de una planta de procesado de cerdo Ibérico (Ortiz et al., 2010). Las cepas se mantuvieron a -80 ºC en caldo BHI (*Brain Heart Infusion*, Biolife S.r.l., Milán, Italia). Antes de su uso en los experimentos, se cultivaron por separado en BHI a 37 ºC durante 18 h.

1.2. Concentración mínima inhibitoria

Se determinó la concentración mínima inhibitoria (CMI) del timol (T, ≥ 99% pureza, Sigma-Aldrich, St. Louis, EE. UU.), carvacrol (CV, > 98% pureza, Sigma-Aldrich), y cinamaldehído (CN, > 95% pureza, Sigma-Aldrich), frente a *L. monocytogenes* en caldo BHI. Para ello, se preparó una solución inicial 10 mg/mL de cada uno de los AEs en agua destilada. Se realizaron diluciones seriadas en placas multipocillo a concentraciones finales de 2,5, 1,25, 0,63, 0,31 y 0,16 mg/mL. Seguidamente, se preparó un cóctel con las cepas S2, S4-2, S7-2 y S12-1 de *L. monocytogenes* en caldo BHI a doble concentración, mezclando a partes iguales cada uno de los cultivos *overnight* independientes. Las placas se inocularon con el cóctel a una concentración aproximada de 10^6 UFC/mL y se incubaron a 37 ºC durante 24 h en un

espectrofotómetro automatizado (Bioscreen C, Bio-Rad Laboratories S.A., Madrid, España). La densidad óptica de los cultivos se midió cada 30 min a una longitud de onda de 600 nm (DO_{600}). Se utilizó caldo BHI como control negativo y caldo BHI inoculado con el cóctel de *L. monocytogenes* como control positivo. La CMI se definió como la concentración más baja de los AEs que inhibió el crecimiento del patógeno. Además de la CMI, se utilizó herramienta de Excel DMFit para ajustar los datos de crecimiento de *L. monocytogenes* y determinar la tasa máxima de crecimiento (μ_{max}) y la fase de latencia (λ) para cada uno de los AEs. Se realizaron dos ensayos independientes con muestras triplicadas.

1.3. Actividad antimicrobiana de AEs en jamón curado loncheado

Se pesaron muestras de 10 g de jamón curado loncheado en el laboratorio a partir de un jamón comercial, y se inocularon en superficie con el cóctel de *L. monocytogenes* indicado anteriormente a una concentración final aproximada de 10^6 UFC/g. Las muestras se mantuvieron a 4 ºC durante 2 h para favorecer la adsorción del patógeno al músculo. Asimismo, se preparó una solución inicial de 125 mg/mL de T, CV y CN en agua destilada. Los AEs se añadieron en la superficie del jamón a una concentración final de 1,25 mg/g. Las muestras se envasaron al vacío y se mantuvieron a 4 y 12 ºC durante 30 d. Se incluyeron muestras de jamón curado loncheado inoculadas con *L. monocytogenes* y sin AEs como control positivo. El ensayo se realizó por duplicado.

1.4. Recuentos de *L. monocytogenes*

Las muestras se homogenizaron en agua de peptona estéril al 0,1% (peso/volumen) durante 120 s en un homogenizador Silver Masticator (IUL Instruments, Barcelona, España). Se prepararon diluciones decimales seriadas en la misma solución y los recuentos de *L. monocytogenes* se realizaron en placas duplicadas de CHROMagar *Listeria* (CH-L, Scharlab S.L., Barcelona, España) incubadas durante 48 h a 37 ºC. Las muestras se analizaron a las 0 h y 2, 7, 15 y 30 d tras la aplicación de los AEs.

1.5. Análisis estadístico

Se realizó un análisis de varianza (ANOVA) con tratamiento, temperatura y tiempo de refrigeración como principales efectos. Se utilizó el test de Tukey para determinar diferencias significativas entre medias para un intervalo de confianza del 95% (Steel et al., 1996).

2. Resultados

2.1. Concentración mínima inhibitoria

En la Figura 7 se muestran las curvas de crecimiento de *L. monocytogenes* con concentraciones crecientes de T, CV y CN. Los tres AEs presentaron una actividad inhibitoria similar frente al patógeno con una CMI de 1,25 mg/mL.

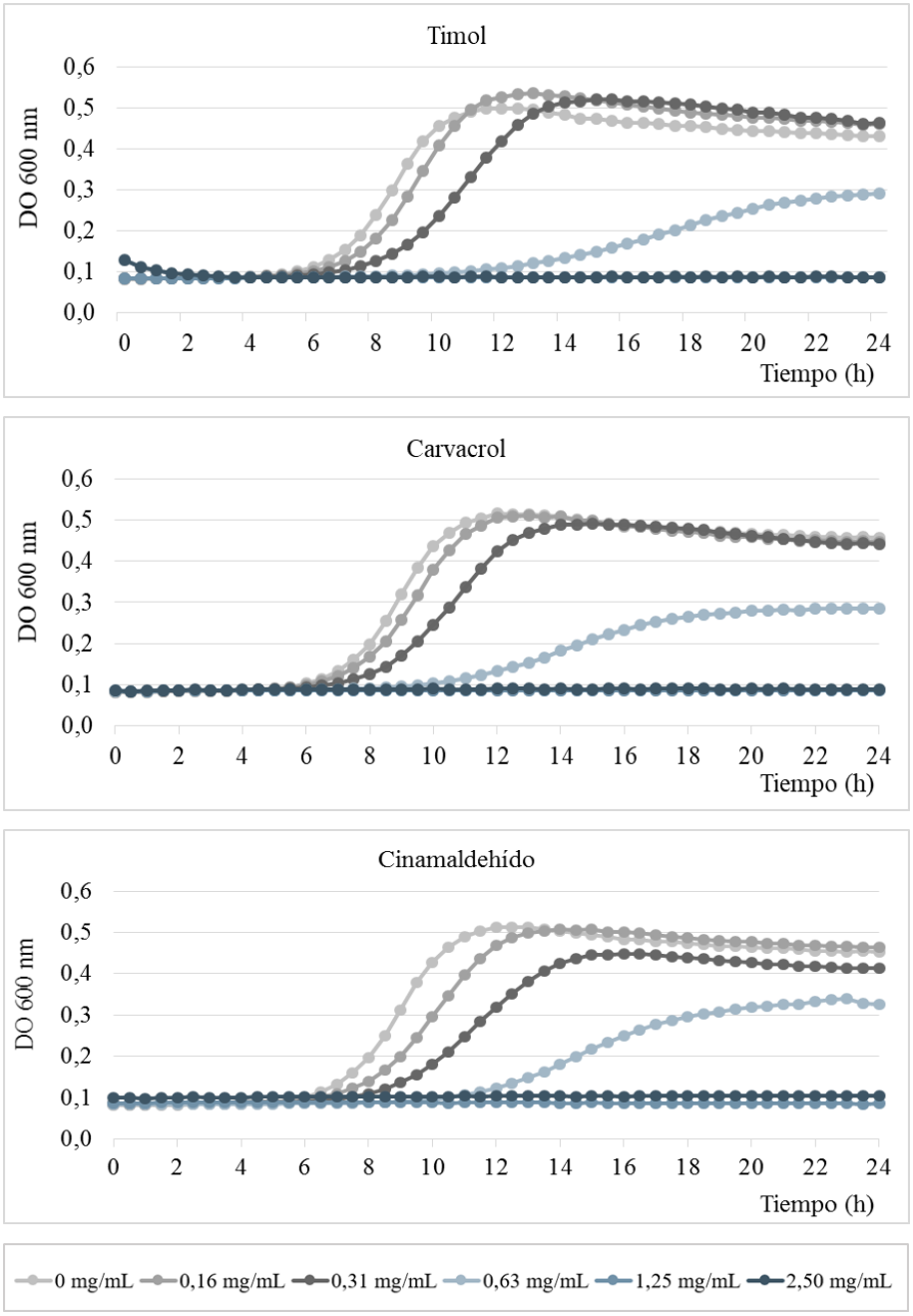

Figura 7. Concentración mínima inhibitoria (CMI) de tres aceites esenciales (AEs) frente a un cóctel de cuatro cepas de *L. monocytogenes* en caldo BHI a 37 ºC durante 24 horas.

Los datos de crecimiento se ajustaron con la herramienta de Excel DMFit para determinar la tasa de crecimiento (μ_{max}) y fase de latencia (λ) de *L. monocytogenes* en caldo BHI (Tabla 2). La tasa de crecimiento de *L. monocytogenes* se redujo significativamente ($P < 0,01$) con concentraciones iguales o superiores a 0,31 mg/mL de T, y 0,63 mg/mL de CV y CN. La fase de latencia del patógeno aumentó significativamente ($P < 0,05$) con concentraciones de T iguales a 0,31 mg/mL, y 0,63 mg/mL de CN y CV.

Tabla 2. Tasa máxima de crecimiento (μ_{max}) y fase de latencia (λ) de *L. monocytogenes* en caldo BHI en presencia de distintas concentraciones de tres AEs.

	Concentración (mg/mL)	μ_{max} (h^{-1})	λ (h)	R^2
T	0	0,121 ± 0,005c	6,67 ± 0,18a	0,988
	0,16	0,122 ± 0,009c	7,36 ± 0,27a	0,990
	0,31	0,095 ± 0,007b	8,45 ± 0,42bc	0,986
	0,63	0,018 ± 0,006a	11,84 ± 2,18c	0,991
	1,25	0	-	-
	2,50	0	-	-
CV	0	0,125 ± 0,012b	7,12 ± 0,26a	0,992
	0,16	0,119 ± 0,006b	7,54 ± 0,47a	0,994
	0,31	0,094 ± 0,007b	8,33 ± 0,60a	0,989
	0,63	0,026 ± 0,016a	10,17 ± 0,20b	0,991
	1,25	0	-	-
	2,50	0	-	-
CN	0	0,122 ± 0,002b	7,10 ± 0,29a	0,992
	0,16	0,102 ± 0,012b	7,98 ± 0,37a	0,994
	0,31	0,077 ± 0,011ab	8,98 ± 0,86ab	0,993
	0,63	0,043 ± 0,025a	12,94 ± 2,71b	0,994
	1,25	0	-	-
	2,50	0	-	-

μ_{max}, tasa máxima de crecimiento; λ, fase de latencia; R^2, coeficiente de determinación. T: timol; CV: carvacrol; CN: cinamaldehído. Los valores representan la media ± desviación estándar de muestras triplicadas de dos experimentos independientes. Los valores dentro de la misma columna con distinta letra difieren significativamente ($P < 0,05$) para cada tratamiento.

2.2. Actividad antimicrobiana de AEs frente a *L. monocytogenes* en jamón curado loncheado

Los recuentos de *L. monocytogenes* en jamón curado loncheado con T, CV y CN mantenido a 4 y 12 ºC durante 30 d se recogen en la Tabla 3. Los recuentos iniciales en jamón control fueron 5,91 log UFC/g. A los 2 d, se observaron reducciones significativas ($P < 0,001$) pero inferiores a 1 unidad logarítmica en jamón curado loncheado con los tres AEs a 4 y 12 ºC.

Tabla 3. *L. monocytogenes* (log UFC/g) en jamón curado loncheado tratado con tres AEs a una concentración de 1,25 mg/g durante 30 días a 4 y 12 ºC.

		Tiempo (días)			
		2	7	15	30
4 ºC	C	5,69 ± 0,15bAB	5,95 ± 0,15bB	5,48 ± 0,09aA	5,64 ± 0,15cA
	T	5,72 ± 0,07bB	5,52 ± 0,13abAB	5,63 ± 0,14aB	5,23 ± 0,21abA
	CV	5,43 ± 0,07aAB	5,20 ± 0,36aAB	5,51 ± 0,09aB	5,02 ± 0,18aA
	CN	5,63 ± 0,14abA	5,58 ± 0,15abA	5,57 ± 0,09aA	5,49 ± 0,14bcA
12 ºC	C	5,89 ± 0,04cA	5,63 ± 0,19aA	5,60 ± 0,22aA	5,62 ± 0,09bA
	T	5,29 ± 0,06aA	5,45 ± 0,08aB	5,49 ± 0,02aB	5,37 ± 0,06abAB
	CV	5,55 ± 0,14bA	5,53 ± 0,07aA	5,38 ± 0,12aA	5,34 ± 0,09abA
	CN	5,63 ± 0,07bB	5,44 ± 0,02aAB	5,51 ± 0,04aB	5,10 ± 0,33aA

C: control; T: timol; CV: carvacrol; CN: cinamaldehído. Recuentos iniciales de *L. monocytogenes* (0 h) en jamón control: 5,91 ± 0,04 log UFC/g. Los valores representan la media ± desviación estándar de dos experimentos independientes. Los valores en la misma columna con diferente letra minúscula difieren significativamente ($P < 0,05$) para la temperatura descrita. Los valores en la misma fila con distinta letra mayúscula difieren significativamente ($P < 0,05$).

Durante los 30 d de refrigeración, los recuentos de *L. monocytogenes* en jamón control a 4 y 12 ºC disminuyeron ligeramente. Asimismo, se observó una disminución de los niveles del patógeno en jamón tratado con AEs. Tras 30 de refrigeración a 4 ºC, los valores de *L. monocytogenes* en jamón con CV fueron 0,9 unidades logarítmicas inferiores a los observados en jamón control a tiempo 0. A 12 ºC, las mayores reducciones se registraron en jamón con CN, con recuentos 0,8 unidades logarítmicas inferiores a los

registrados inicialmente en jamón curado control. La temperatura no afectó significativamente a los recuentos del patógeno.

3. Conclusión

La actividad antimicrobiana de los tres AEs fue reducida, con valores de inactivación de *L. monocytogenes* inferiores a una unidad logarítmica en el jamón curado. Con el fin de evaluar su efecto antimicrobiano en combinación con altas presiones, se seleccionó el timol ya que modificó en menor medida que el carvacrol y el cinamaldehído el olor y aspecto del producto.

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ORIGINAL PAPER

Combined Effect of High Pressure Processing with Enterocins or Thymol on the Inactivation of *Listeria monocytogenes* and the Characteristics of Sliced Dry-cured Ham

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Abstract
The effect of high pressure processing (HPP) at 450 MPa for 10 min, enterocins A and B, thymol, and their combinations on the inactivation of a four-strain cocktail of *Listeria monocytogenes* and the properties of sliced dry-cured ham during 30 days at 4 and 12 °C was investigated. Enterocins A and B initially reduced *L. monocytogenes* levels by more than 2.5 log units, but a regrowth was recorded during the storage. Individual treatments of thymol and HPP exhibited a low antimicrobial effect against the pathogen. A synergistic antibacterial activity against *L. monocytogenes* was observed when HPP was combined with enterocins A and B, preventing the recovery of the pathogen during all the storage period. Such combined treatment also maintained total viable counts (TVC) at low levels after 30 days at 4 and 12 °C. Minor changes were detected in pH, a_w, color parameters, and shear strength values in dry-cured ham treated with enterocins A and B, thymol, HPP, and their combinations during the storage at both temperatures. Combination of HPP at 450 MPa for 10 min and enterocins A and B might be applied as a hurdle technology, since it reduced *L. monocytogenes* counts and spoilage bacteria, and slightly affected the characteristics of sliced dry-cured ham.

Keywords *L. monocytogenes* · Enterocins · Thymol · High pressure processing · Sliced dry-cured ham

Introduction

Nowadays, there is a growing trend among consumers in minimally processed, fresh, healthy, and safe ready-to-eat (RTE) foods. Arising from this, a large variety of RTE products are available in the market, including meat products such as sliced dry-cured ham, which can be contaminated with food-borne pathogens during the industrial processing. One of the most relevant pathogens in RTE products is *Listeria monocytogenes*, a Gram-positive bacteria which can cause a severe food-borne disease called listeriosis, mainly in neonates, pregnant women, elderly, and immunocompromised individuals. *L. monocytogenes* is among the most important causes of death from food-borne infections in Europe, with a fatality rate of 16.2% during 2016 (EFSA 2017). This pathogen is able to grow well in a wide variety of foods (e.g., deli meats, dairy products, smoked fish, vegetables), even under different stress conditions such as refrigeration temperatures, low pH, or high levels of NaCl (Lado and Yousef 2007). Moreover, *L. monocytogenes* can persist in the food-processing environment for a long time due to its ability to form biofilms and to resist cleaning and disinfection processes, increasing the risk of food product contamination.

Sliced dry-cured ham can be contaminated with *L. monocytogenes*, mainly during slicing, packaging, and other handling processes (Lin et al. 2006), which may be allowed to multiply owing to inadequate storage temperatures. Food processors have implemented a number of strategies to control food-borne pathogens, including non-thermal technologies such as high pressure processing (HPP) (Aymerich et al. 2008), addition of organic acids (Geornaras et al. 2005), or biopreservatives like nisin (Delves-Broughton et al. 1996). HPP is an effective non-thermal technology applied to control post-processing contaminants in many foods. Different pressurized products are usually commercialized in the international market, including RTE meat products such as sliced cooked ham, turkey or chicken cuts, dry-cured ham, frankfurters, or fermented meat products. The antimicrobial activity of HPP treatments (400–600 MPa) against *L. monocytogenes* has been widely reported in RTE food products (Aymerich et al. 2005; Hereu et al. 2012; Montiel et al. 2012; Montiel et al. 2015).

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The control of food-borne pathogens has also been carried out by biopreservation. The application of lactic acid bacteria (LAB) or their metabolites inhibited the growth of *L. monocytogenes* in milk and dairy products (Arqués et al. 2008a, b), vegetables (Huang et al. 2009), meats (Nieto-Lozano et al. 2010; Garriga et al. 2002), or seafood products (Tahiri et al. 2009; Ghanbari et al. 2013). Specifically, bacteriocins such as enterocins, produced by *Enterococcus* spp. and ascribed to class II, have shown antimicrobial activity against *L. monocytogenes* in cooked ham (Marcos et al. 2008). Other biopreservatives, like essential oils (EOs), have been considered as a new and safe approach to inhibit the growth of food-borne pathogens and spoilage bacteria (Raeisi et al. 2016). *L. monocytogenes* has been shown to be especially sensitive to essential oils (Friedly et al. 2009), as well as other food-borne pathogens including *Staphylococcus aureus*, *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Campylobacter* spp. (Callaway et al. 2011). Thymol (2-isopropyl-5-methylphenol) is the main monoterpene phenol found in the EOs extracted from plants belonging to the *Lamiaceae* family (Marchese et al. 2016; Licata et al. 2015; Mancini et al. 2015) and it is permitted for direct addition to human food by the United States Food and Drug Administration (FDA 2017).

Combining HPP with a biopreservation method could achieve a stronger antimicrobial effect, allowing the use of lower pressurization intensities and additive concentrations. Combined treatments may provide greater protection against *L. monocytogenes*, improving the safety of RTE meat products. The main aim of this work was to evaluate the antimicrobial effect of HPP combined with an extract of enterocins A and B (ENT) or thymol (THY) on *L. monocytogenes* in sliced dry-cured ham stored under strict refrigeration temperature (4 °C) and temperature abuse conditions (12 °C) for 30 days. The effect on total viable counts and the physicochemical, color, and rheological characteristics of sliced dry-cured ham were also investigated.

Materials and Methods

Microorganisms

L. monocytogenes S2 (serotype 1/2a), S4-2 (serotype 1/2b), S7-2 (serotype 4b), and S12-1 (serotype 1/2c) (from the INIA Culture Collection, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid, Spain), isolated from an Iberian pig processing plant and previously characterized by Ortiz et al. (2010) were used as test organisms. The strains were maintained at – 80 °C in brain heart infusion broth (BHI, Biolife S.r.l., Milano, Italy) with 20% glycerol. A four-strain cocktail of *L. monocytogenes* was prepared by mixing equal amounts of cultures of *L. monocytogenes* strains, separately grown in BHI for 18 h at 37 °C. Enterocins A and B producing *Enterococcus faecium* INIA TAB7 (Rodríguez et al. 2000) was used for enterocins extract production. The strain was maintained as stock culture at − 80 °C in De Man, Rogosa and Sharpe broth (MRS, Biolife) supplemented with 20% glycerol.

Dry-cured Ham Samples

Dry-cured ham obtained from a commercial supplier in Spain was aseptically sliced in the laboratory. Slices were divided into 10-g pieces for microbiological analysis and 70-g pieces for pH, water activity (a_w), color, and texture determinations. Half of the 10-g samples were inoculated by spreading 100 μl of *L. monocytogenes* strains on the surface of dry-cured ham in order to achieve a final population of approximately 10^6 cfu/g, and the other half, not inoculated, were used to determine total viable counts (TVC). The 70-g samples were not inoculated and were used for the determination of pH, a_w, color, and texture. Samples were divided into six groups: control (non-pressurized and without antimicrobials), ENT (with enterocins A and B extract), THY (with thymol), HPP (pressurized), ENT + HPP (with enterocins A and B extract added before the pressurization), and THY + HPP (with thymol added before the pressurization).

Antimicrobials

E. faecium INIA TAB7 was grown in MRS broth with Tween® 80 for 18 h at 30 °C and used to obtain the enterocins A and B extract as previously described (Garriga et al. 2002). The antimicrobial activity was measured against the cocktail of *L. monocytogenes* strains through the agar spot test (Barefoot and Klaenhammer 1983) and expressed in arbitrary units (AU) per ml. Enterocins A and B extract was added on the surface of sliced dry-cured ham to achieve a final activity of 1054 AU/g.

Thymol (≥ 99% purity, Sigma-Aldrich, St. Louis, USA) was prepared in distilled deionized water at 125 mg/ml. EO solution was added on the surface of sliced dry-cured ham at a final concentration of 1.25 mg/g.

Pressurization of Samples

Inoculated and non-inoculated samples were individually vacuum-packaged in high-barrier multilayer bags (200 mm × 300 mm, BB325, Cryovac Sealed Air Corporation, Milan, Italy) and pressurized at 450 MPa for 10 min. High pressure processing was carried out in a 135-l-capacity isostatic press (NC, Hiperbaric, Burgos, Spain). The compression rate was approximately 225 MPa/min and the pressure release time was 7 s. The water temperature used as pressure-transmitting medium was 18 °C. After treatment, pressurized and non-

pressurized samples were stored under refrigeration at 4 °C and 12 °C during 30 days. Two independent trials were carried out.

Microbiological Analysis

Inoculated and non-inoculated samples of dry-cured ham were analyzed at 1, 7, 15, and 30 days after pressurization. Samples (10 g) were diluted tenfold with sterile 0.1% (*w*/*v*) peptone water solution in stomacher bags and homogenized for 180 s using a Silver Masticator homogenizer (IUL Instruments, Barcelona, Spain). Decimal dilutions of the homogenates were prepared in the same sterile solution. *L. monocytogenes* counts were determined on duplicate plates of CHROMagar *Listeria* (CH-L, Scharlab S.l., Barcelona, Spain) incubated at 37 °C for 48 h. TVC counts were determined on duplicate plates of general medium plate count agar (PCA, Liofilchem S.r.l., Roseto degli Abruzzi, Italy) incubated at 30 °C for 72 h.

pH and a_w

Non-inoculated samples (5 g) were homogenized with 15 ml of distilled water in stomacher bags for 180 s and pH determinations were made using a pH meter GLP22 (Crison Instruments S.A., Barcelona, Spain). Three measurements were taken per sample. Water activity (a_w) was measured in dry-cured ham samples using an Aqualab Series 3 water activity meter (Decagon Devices Inc., Washington, USA). Three measurements were performed per sample. pH and a_w determinations were carried out at 1, 15, and 30 days after pressurization.

Color and Texture

Non-inoculated 4-mm-thick slices of dry-cured ham were used to determine color and texture parameters. L^* (lightness, intensity of white color), a^* (+ a, red; − a, green) and b^* (+ b, yellow; − b, blue) values were determined with a chromometer CM-700d (Konica Minolta Ltd., Osaka, Japan) in different locations of the dry-cured ham slices, and the respective mean of nine measurements per sample was expressed as the final value. The analysis was carried out with duplicate samples at 1, 15, and 30 days after pressurization.

Dry-cured ham texture was measured using an Instron Universal testing Machine 4301 (Instron Ltd., Barcelona, Spain), controlled by the Bluehill® v2.0 software and a crosshead speed of 100 mm/min. The force required to shear through the sample was determined using a V-shaped cutting Warner-Bratzler blade with samples cut into 5 × 3 × 0.4-cm pieces. The analysis was carried out with duplicate samples at room temperature at 1, 15, and 30 days after pressurization, and at least six measurements were taken from each sample.

Statistical Analysis

Analysis of variance (ANOVA) with treatment, time of refrigeration, and temperature of refrigeration as main effects was carried out by means of SPSS Statistics 22.0 software (IBM Corp., Armonk, NY, USA). Significant differences between means were assessed by Tukey's test for a confidence interval of 95% (Steel et al. 1996).

Results and Discussion

L. monocytogenes

L. monocytogenes counts in control and treated samples of sliced dry-cured ham during refrigerated storage at 4 and 12 °C are shown in Table 1. *L. monocytogenes* initial counts in control dry-cured ham were 6.60 and 6.67 log cfu/g at 4 and 12 °C, respectively. One day after treatments, reductions of 2.54 and 2.95 log units at 4 and 12 °C, respectively, were registered in dry-cured ham with enterocins A and B, whereas the antilisterial effect of thymol or HPP was scarce. The antimicrobial activity of HPP increased significantly when it was applied in combination with enterocins A and B, with levels 4.38 and 5.25 log cfu/g lower than those observed in control samples at 4 and 12 °C, respectively. During refrigerated storage, a slight decrease in *L. monocytogenes* levels was recorded in control samples and in those individually treated with thymol or HPP. A bacteriostatic effect was recorded against the pathogen in enterocins A- and B-treated samples within the first 7 days, but *L. monocytogenes* restarted its growth from day 15 onwards. The combination of enterocins A and B and HPP avoided the recovery of the pathogen during all the storage period.

Although dry-cured ham is considered a risk-free RTE meat product, pathogens as *L. monocytogenes* can overcome the stressful conditions encountered in sliced dry-cured ham and be able to multiply in case of temperature abuse during storage. Enterocins represent a promising tool to control, as a part of a multi-hurdle preservation system, the growth of *L. monocytogenes* in foods (Herranz et al. 2001). Enterocin L50A and enterocin L50B at 400 AU/ml prevented the growth of *L. monocytogenes* in deli ham for at least 10 weeks at 8 °C and 30 days at 15 °C (Du et al. 2017). In the present work, enterocins A and B reduced *L. monocytogenes* counts more than 2.5 log units, maintaining the levels 2.08 and 1.95 log units below control samples after 30 days of storage at 4 and 12 °C, respectively.

The effectiveness of thymol and thymol-rich EOs to control *L. monocytogenes* has been widely investigated in vitro (Mith et al. 2014) and in different food products as milk (Pan et al. 2014) or meat products like beef processed by "sous vide cook-chill" (Gouveia et al. 2017) and marinated beef (Moon

Table 1 *L. monocytogenes* counts (log cfu/g) in sliced dry-cured ham treated with enterocins A and B (ENT, 1054 AU/g), thymol (THY, 1.25 mg/g), high pressure processing (HPP, 450 MPa/10 min), and their combinations during 30 days at 4 and 12 °C

		1 day	7 days	15 days	30 days
4 °C	Control	6.60 ± 0.11 dB	6.61 ± 0.05eB	6.35 ± 0.03dA	6.29 ± 0.03dA
	ENT	4.06 ± 0.23bA	3.67 ± 0.07bA	4.88 ± 0.42bB	4.21 ± 0.38bA
	THY	5.94 ± 0.05cC	5.92 ± 0.11cdBC	5.68 ± 0.03cA	5.77 ± 0.11cdAB
	HPP	6.15 ± 0.06cB	6.12 ± 0.12 dB	5.81 ± 0.09cA	6.01 ± 0.05 dB
	ENT + HPP	2.22 ± 0.38aA	1.89 ± 0.16aA	2.91 ± 0.39aA	2.80 ± 1.44aA
	THY + HPP	5.96 ± 0.01cC	5.68 ± 0.21cB	5.66 ± 0.05cB	4.99 ± 0.11bcA
12 °C	Control	6.67 ± 0.16 dB	6.53 ± 0.18eAB	6.57 ± 0.04dAB	6.33 ± 0.02dA
	ENT	3.72 ± 0.24bA	3.69 ± 0.06bA	4.27 ± 0.27bB	4.38 ± 0.33bB
	THY	6.09 ± 0.07cdC	5.98 ± 0.03dBC	5.79 ± 0.14cdAB	5.57 ± 0.16cA
	HPP	6.06 ± 0.05cdA	5.85 ± 0.22dA	5.96 ± 0.08cdA	5.90 ± 0.12cdA
	ENT + HPP	1.42 ± 0.60aA	1.75 ± 0.64aA	2.13 ± 1.17aA	3.25 ± 0.02aA
	THY + HPP	5.48 ± 0.48cAB	5.20 ± 0.21cAB	5.53 ± 0.15cB	4.88 ± 0.27bA

L. monocytogenes initial counts (0 h) in control sliced dry-cured ham were 6.60 log cfu/g

Values are the mean ± SD. Means within the same column with different lowercase letters differ significantly at $P < 0.05$ for a given temperature. Means within the same row with different uppercase letters differ significantly at $P < 0.05$

et al. 2017), among others. In the present work, antilisterial activity of thymol was scarce with reductions ranging between 0.5 and 0.8 log units. Pesavento et al. (2015) reported higher antimicrobial effect against *L. monocytogenes* with thyme EO (43.1% of thymol) at different concentrations in minced meat stored at 4 °C. The low bactericidal activity observed in the present study may be due to the hydrophobicity of thymol, which could bind to other hydrophobic components of dry-cured ham such as fat, hindering its uniform distribution in the food matrix (Pesavento et al. 2015; Shah et al. 2012).

The efficacy of HPP to eliminate *L. monocytogenes* in meat products is affected by intrinsic factors of the food matrix. *L. monocytogenes* levels diminished 3.85 log units when HPP treatments of 600 MPa were applied for 5 min to dry-cured ham with a_w of 0.92, whereas reductions of 1.85 log units were obtained with a_w of 0.88 (Hereu et al. 2012). Low-intensity treatment applied in the present work of 450 MPa for 10 min diminished the pathogen levels less than 1.0 log unit due to the low a_w of the dry-cured ham used in the experiments, with an average value of 0.874. The combination of HPP with other preservative hurdles could be recommendable in products with low water content to provide greater protection than individual treatments. De Alba et al. (2015) reported similar reductions of *L. monocytogenes* in dry-cured ham pressurized at 450 MPa, but the antimicrobial effect was not improved in combination with the lactoperoxidase system activation or lactoferrin. The application of bacteriocin-producing lactic acid bacteria or bacteriocins with HPP treatments has shown a strong cooperative effect on *L. monocytogenes* inactivation in cheese (Arqués et al. 2005), fermented sausages (Ananou et al. 2010), sliced cooked ham (Liu et al. 2012), or sliced dry-cured ham (Hereu et al. 2012). Jofré et al. (2008) observed that the combination of semi-purified enterocins A and B and HPP (600 MPa for 5 min) achieved reductions of 2.52 log units in dry-cured ham 1 day after treatments and the absence of *L. monocytogenes* was registered after 5 days of storage at 4 °C. In this work, the combination of HPP and enterocins A and B exhibited a synergistic bactericidal effect against *L. monocytogenes* in dry-cured ham during 30 days of storage at normal or abuse refrigeration temperatures. Concerning the combination of thymol and HPP to control *L. monocytogenes*, few studies have been published. Karatzas et al. (2001) noticed that combined treatments of thymol and HPP showed a synergistic effect against *L. monocytogenes* in both culture medium and semi-skimmed milk. In our work, this combined treatment kept the pathogen levels 1.30 and 1.45 log units lower than those observed in control samples at 4 °C and 12 °C, respectively, after 30 days of storage. High concentrations of thymol could be required in foods to control *L. monocytogenes* and this may exceed the threshold of acceptability for the taste of food (Pesavento et al. 2015). Its combination with HPP achieved acceptable reductions in *L. monocytogenes* counts using lower concentrations of this EO. However, the presence of thymol affected the odor of dry-cured ham, although odor intensity tended to attenuate throughout the refrigerated storage.

Total Viable Counts

The mean total viable counts (TVC) in control and treated samples of sliced dry-cured ham during refrigerated storage at 4 and 12 °C are given in Table 2. Initial microbial counts slightly increased in control samples kept at 4 °C, not exceeding 4 log units after 30 days, whereas under abuse temperature conditions (12 °C), TVC reached levels close to 6 log cfu/g.

Table 2 Total viable counts (log cfu/g) in sliced dry-cured ham treated with enterocins A and B (ENT, 1054 AU/g), thymol (THY, 1.25 mg/g), high pressure processing (HPP, 450 MPa/10 min), and their combinations during 30 days at 4 and 12 °C

		1 day	7 days	15 days	30 days
4 °C	Control	2.70 ± 0.31bcA	3.49 ± 0.07cA	3.15 ± 0.30bA	3.42 ± 0.68bA
	ENT	3.08 ± 0.10bcA	3.59 ± 0.13cB	3.35 ± 0.25bAB	3.63 ± 0.12bB
	THY	3.47 ± 0.08cA	3.32 ± 0.07cA	3.52 ± 0.49bA	3.79 ± 0.36bA
	HPP	2.23 ± 1.06bA	2.08 ± 0.15bA	2.49 ± 0.58abA	2.36 ± 0.57aA
	ENT + HPP	< 1.00aA	< 1.00aA	1.85 ± 0.00aB	2.03 ± 0.05aC
	THY + HPP	2.20 ± 0.17bA	2.54 ± 0.62bA	1.76 ± 0.40aA	2.00 ± 0.00aA
12 °C	Control	3.48 ± 0.42cA	3.19 ± 0.48cdA	5.02 ± 0.17cB	5.46 ± 0.08eB
	ENT	3.26 ± 0.07bcA	3.96 ± 0.28 dB	4.99 ± 0.09cC	5.04 ± 0.05dC
	THY	2.96 ± 0.25bcA	4.08 ± 0.02 dB	4.65 ± 0.18cC	5.34 ± 0.09eD
	HPP	1.59 ± 0.83aA	1.59 ± 0.83abA	2.04 ± 0.06bA	3.84 ± 0.00cA
	ENT + HPP	2.42 ± 0.51abB	< 1.00aA	< 1.00aA	1.77 ± 0.10aAB
	THY + HPP	3.00 ± 0.34bcAB	2.45 ± 0.17bcA	2.34 ± 0.54bA	3.58 ± 0.07bB

Values are the mean ± SD. Means within the same column with different lowercase letters differ significantly at $P < 0.05$ for a given temperature. Means within the same row with different uppercase letters differ significantly at $P < 0.05$

One day after treatments, only the combination of enterocins A and B and HPP significantly reduced TVC in dry-cured ham, with counts < 1.00 and 1.60 log cfu/g at 4 and 12 °C, respectively. During the storage, individual pressure treatments avoided the growth of TVC during 30 and 15 days of storage at 4 and 12 °C, respectively. Its combination with enterocins A and B diminished TVC within the first days of storage, keeping microbial counts at or below 2 log units at the end of storage at both temperatures assayed.

Microorganisms cause food spoilage through the growth and active metabolism of food components or, even in the absence of live cells, by their extracellular and intracellular enzymes, which react with food components and change their functional properties leading to spoilage (Ray 2004). TVC are used as indicators to assess the quality of food products and determine their shelf life throughout storage (Park and Kim 2013). In low water activity and high salt content foods as dry-cured ham, the predominant spoilage microorganisms are Gram-positive, catalase-positive cocci, yeasts, and molds, mainly on the surface of whole ham (Rodríguez et al. 1998). During handling processes, such as deboning, slicing, or packaging, they can be transferred to the final sliced product. HPP is an important technology for the meat industry to control not only food-borne pathogens but also spoilage microorganisms. Garriga et al. (2004) observed that aerobic total counts were maintained below 3.50 log units during 120 days when sliced vacuum-packed dry-cured ham was pressurized at 600 MPa for 6 min and stored at 4 °C. In this work, HPP at 450 MPa for 10 min kept the TVC below 3 and 4 log units in sliced dry-cured ham maintained at 4 and 12 °C, respectively, during 30 days.

To our knowledge, there are not published data about the effect of bacteriocins, EOs, and its combination with HPP on TVC in sliced dry-cured ham. In our work, TVC in dry-cured ham treated with enterocins A and B or thymol did not differ from those registered in control samples during 30 days of storage. However, the two combined treatments maintained TVC below 2.10 and 3.60 log units after 30 days at 4 and 12 °C, respectively. Thus, the combination of HPP with natural preservation methods, such as bacteriocins or EOs, may provide a greater protection, improving the safety and quality of meat products and extending their shelf life.

pH and a_w

Values of pH and water activity during refrigerated storage of control and treated sliced dry-cured ham with enterocins A and B, thymol, and HPP applied individually and in combination are shown in Table 3. One day after treatments, pH values in control dry-cured ham were 5.74 and 5.54 at 4 and 12 °C, respectively, and ranged from 5.55 to 5.76 in samples treated with enterocins A and B, thymol, HPP, and their combinations. Values of pH kept quite stable in control dry-cured ham throughout the storage at both temperatures assayed, whereas a slight increase was observed in all treated samples, with values ranging from 5.71 to 5.95. HPP treatment caused the highest increases in pH, as previously observed in pressurized dry-cured ham (De Alba et al. 2013), which could be attributed to protein denaturation or loss of protons caused by treatment (Marcos et al. 2005).

Values of a_w at day 1 in control dry-cured ham were 0.873 and 0.868 at 4 and 12 °C, respectively. In samples treated with thymol, individually or in combination with HPP, values were significantly higher than in control dry-cured ham at both temperatures assayed. During the storage, a_w values increased in dry-cured ham treated with enterocins A and B, HPP, and their combination, ranging from 0.875 to 0.878 after 30 days at 4 and 12 °C. Despite the treatments and storage time

Table 3 Values of pH and a_w in sliced dry-cured ham treated with enterocins A and B (ENT, 1054 AU/g), thymol (THY, 1.25 mg/g), high pressure processing (HPP, 450 MPa/ 10 min), and their combinations during 30 days at 4 and 12 °C

			1 day	15 days	30 days
pH	4 °C	Control	5.74 ± 0.02bA	5.79 ± 0.04aA	5.71 ± 0.03aA
		ENT	5.73 ± 0.03bA	6.00 ± 0.07 dB	5.83 ± 0.03abA
		THY	5.70 ± 0.05bA	5.91 ± 0.04bcdC	5.80 ± 0.03abB
		HPP	5.76 ± 0.04bA	5.95 ± 0.03cdB	5.93 ± 0.08bB
		ENT + HPP	5.67 ± 0.04abA	5.84 ± 0.04abB	5.80 ± 0.09abAB
		THY + HPP	5.58 ± 0.01aA	5.84 ± 0.02abcB	5.86 ± 0.05abB
	12 °C	Control	5.54 ± 0.08aA	5.81 ± 0.02aB	5.61 ± 0.03aA
		ENT	5.62 ± 0.06aA	5.80 ± 0.01aB	5.71 ± 0.01bAB
		THY	5.69 ± 0.02aA	5.91 ± 0.04bC	5.81 ± 0.00cB
		HPP	5.63 ± 0.09aA	5.79 ± 0.02aB	5.87 ± 0.01 dB
		ENT + HPP	5.55 ± 0.02aA	5.79 ± 0.06aB	5.73 ± 0.03bB
		THY + HPP	5.63 ± 0.02aA	5.74 ± 0.01aB	5.80 ± 0.00cC
a_w	4 °C	Control	0.873 ± 0.001abB	0.872 ± 0.001aAB	0.870 ± 0.001aA
		ENT	0.873 ± 0.002abA	0.876 ± 0.001bB	0.875 ± 0.001bAB
		THY	0.875 ± 0.001bcA	0.877 ± 0.002bA	0.876 ± 0.001bA
		HPP	0.872 ± 0.001aA	0.877 ± 0.001bB	0.878 ± 0.002bB
		ENT + HPP	0.874 ± 0.001abcA	0.876 ± 0.001bA	0.878 ± 0.001bB
		THY + HPP	0.876 ± 0.002cA	0.878 ± 0.002bA	0.882 ± 0.002cB
	12 °C	Control	0.868 ± 0.005aA	0.871 ± 0.000aA	0.871 ± 0.001aA
		ENT	0.867 ± 0.000aA	0.875 ± 0.000bB	0.876 ± 0.002abB
		THY	0.869 ± 0.003aA	0.878 ± 0.001cB	0.876 ± 0.001bB
		HPP	0.870 ± 0.002aA	0.874 ± 0.001bB	0.877 ± 0.001bC
		ENT + HPP	0.868 ± 0.002aA	0.875 ± 0.002bB	0.875 ± 0.003abB
		THY + HPP	0.878 ± 0.001bB	0.873 ± 0.000abA	0.879 ± 0.001bB

Values are the mean ± SD. Means within the same column with different lowercase letters differ significantly at $P < 0.05$ for a given temperature. Means within the same row with different uppercase letters differ significantly at $P < 0.05$

influencing a_w of dry-cured ham, the differences with initial control samples were scarce, not exceeding 0.009 and 0.011 a_w units at 4 and 12 °C, respectively. Low a_w values and/or high solute concentrations have been recognized to exert a baroprotective effect and reduce the extent of bacterial inactivation induced by HPP (Patterson 2005). In our work, sliced dry-cured ham exhibited values of a_w between 0.867 and 0.882, which could make difficult the recovery of sublethally injured cells during the storage of the product.

Color

Changes in color parameters (L^*, a^*, and b^*) during refrigerated storage of control and treated sliced dry-cured ham with enterocins A and B, thymol, and HPP applied individually and in combination are given in Table 4. One day after treatments, L^* values did not show significant differences between control and treated dry-cured ham, kept at 4 or 12 °C. At the end of the time storage, a significant increase of lightness was recorded in samples treated with HPP at 12 °C. Values of a^* (redness) were not affected in samples treated with enterocins A and B, thymol, and HPP, individually or in combination, at 4 and 12 °C. However, after 30 days of storage at 12 °C, a^* values were significantly lower in samples treated with enterocins A and B, HPP, and combined treatments than those observed in control dry-cured ham. The values of b^* (yellowness) at day 1 in control and treated dry-cured ham were not significantly different at both temperatures assayed. At the end of storage, b^* values were higher in samples treated with thymol than in control at 4 °C and lower in HPP samples at 12 °C.

Color is considered one of the most important attributes of food appearance. High pressure treatments induce changes in the color of meat products and such color changes have been attributed to denaturation and reduction of myofibrillar sarcoplasmic proteins (Angsupanich and Ledward 1998; Marcos et al. 2010), suggesting an inverse relationship between variations in lightness and solubility of both types of proteins after HPP treatments (Montiel et al. 2014). In our work, L^* values were slightly, but not significantly, higher in samples subjected to 450 MPa for 10 min, individually or in combination with enterocins A and B and thymol, which could be explained by structural changes in proteins in the surface of dry-cured ham. The red pigments of cured meat products are sensitive to high pressure treatments (Andrés et al. 2006) and redness of dry-

Table 4 Color characteristics of sliced dry-cured ham treated with enterocins A and B (ENT, 1054 AU/g), thymol (THY, 1.25 mg/g), high pressure processing (HPP, 450 MPa/10 min), and their combinations during 30 days at 4 and 12 °C

			1 day	15 days	30 days
*L**	4 °C	Control	34.18 ± 6.84aA	36.47 ± 5.38aA	35.01 ± 4.86aA
		ENT	39.36 ± 5.16aA	35.43 ± 4.98aA	38.57 ± 6.80aA
		THY	36.85 ± 4.95aA	34.67 ± 5.79aA	36.93 ± 4.53aA
		HPP	37.12 ± 5.32aA	36.52 ± 5.39aA	37.78 ± 5.71aA
		ENT + HPP	38.04 ± 5.87aA	38.03 ± 5.60aA	39.45 ± 6.33aA
		THY + HPP	38.76 ± 6.34aA	36.93 ± 6.95aA	38.93 ± 6.52aA
	12 °C	Control	37.72 ± 5.80aA	36.53 ± 6.65aA	36.69 ± 6.77aA
		ENT	35.69 ± 5.79aA	32.82 ± 7.30aA	37.20 ± 4.17aA
		THY	34.79 ± 4.97aA	34.31 ± 5.68aA	39.54 ± 3.80abB
		HPP	38.23 ± 6.09aAB	36.86 ± 7.54aA	42.19 ± 4.93bB
		ENT + HPP	38.69 ± 5.70aA	36.10 ± 6.44aA	39.53 ± 4.25abA
		THY + HPP	38.87 ± 6.09aA	36.40 ± 7.80aA	39.51 ± 5.95abA
*a**	4 °C	Control	11.17 ± 1.77abA	9.99 ± 1.63aA	10.48 ± 1.60abA
		ENT	10.90 ± 2.04abAB	11.77 ± 2.80aB	9.35 ± 2.14aA
		THY	11.53 ± 1.80bA	10.74 ± 2.56aA	11.54 ± 3.06bA
		HPP	9.78 ± 1.47aA	10.14 ± 2.15aA	10.21 ± 1.52abA
		ENT + HPP	9.95 ± 1.81abA	11.31 ± 2.16aA	11.34 ± 2.96abA
		THY + HPP	10.28 ± 1.37abA	10.41 ± 2.07aA	10.03 ± 1.47abA
	12 °C	Control	10.30 ± 2.38aA	11.79 ± 2.62bA	12.42 ± 3.43bA
		ENT	11.89 ± 2.63aA	13.99 ± 2.75cB	9.90 ± 2.24aA
		THY	10.07 ± 2.09aA	12.11 ± 1.38bcB	10.37 ± 1.91abA
		HPP	9.86 ± 1.65aAB	11.52 ± 2.96bB	9.77 ± 1.45aA
		ENT + HPP	10.50 ± 1.72aA	10.67 ± 1.67abA	9.84 ± 2.04aA
		THY + HPP	10.16 ± 2.22aA	8.79 ± 1.49aA	9.73 ± 1.38aA
*b**	4 °C	Control	8.05 ± 2.12aA	6.41 ± 2.10aA	6.14 ± 2.86aA
		ENT	7.37 ± 2.58aAB	9.00 ± 2.99aB	5.86 ± 2.69aA
		THY	8.65 ± 2.75aA	7.42 ± 3.37aA	9.44 ± 3.85bA
		HPP	6.66 ± 1.92aA	7.61 ± 2.42aA	6.91 ± 2.20abA
		ENT + HPP	6.99 ± 2.62aA	7.86 ± 3.20aA	8.55 ± 3.21abA
		THY + HPP	7.77 ± 1.91aA	8.03 ± 2.89aA	8.12 ± 2.30abA
	12 °C	Control	6.77 ± 2.27aA	7.71 ± 4.21abA	9.09 ± 3.75bA
		ENT	8.63 ± 2.69aAB	10.07 ± 2.39bB	7.31 ± 3.19abA
		THY	7.18 ± 2.50aA	9.22 ± 2.84abA	7.57 ± 3.37abA
		HPP	6.66 ± 2.22aA	7.62 ± 2.93abA	5.70 ± 2.04aA
		ENT + HPP	6.66 ± 2.40aA	8.06 ± 2.70abA	6.46 ± 2.98abA
		THY + HPP	7.17 ± 2.09aA	7.05 ± 3.02aA	8.27 ± 3.67abA

Values are the mean ± SD. Means within the same column with different lowercase letters differ significantly at $P < 0.05$ for a given temperature. Means within the same row with different uppercase letters differ significantly at $P < 0.05$

cured ham has exhibited decreases with increasing pressure, especially above 400 MPa (De Alba et al. 2013; Fuentes et al. 2010). However, Clariana et al. (2011) observed that high pressure up to 600 MPa for 6 min at 15 °C maintained color characteristics of dry-cured ham after 50 days of refrigerated storage. Similarly, in our work, reductions in *a** values were not recorded in pressurized samples at 450 MPa for 10 min.

According to our results, enterocins A and B or thymol had no significant effects in color parameters immediately after treatment application, whereas minor changes were registered at the end of the storage time.

The appearance and color of food are the first parameters of quality evaluated by consumers and are thus critical factors for acceptance of the food items (León et al. 2006). In the present work, changes in color properties of treated dry-cured ham were scarce, and the visual appearance of the treated samples would not induce consumer rejection.

Texture

Changes in shear strength values during refrigerated storage of control and treated sliced dry-cured ham with enterocins A and B, thymol, and HPP applied individually and in combination are shown in Table 5. At day 1, no significant differences were detected in shear strength of control and treated dry-cured ham at 4 °C, with values ranging between 39.97 and 51.53 N. However, at 12 °C, an increase was recorded in pressurized samples, reaching 53.89 N. Such changes tended to diminish during the storage and no significant differences were found in shear strength values among control and treated samples after 30 days, with values ranging between 42.98 and 46.49 N.

Besides color, texture is also a very important quality characteristic of meat products, which could be affected by the application of natural preservatives or pressure treatments. High pressure induces alteration in meat structure and water distribution, which affect juiciness and increase tenderness, depending on the product composition, the rigor state of the meat, the pressure level, the temperature, and the duration of the treatment (Galazka and Ledward 1998; Ma and Ledward 2004; Sun and Holley 2010) and can be either tenderized or toughened. De Alba et al. (2012) observed a decrease in shear force when 400, 500, or 600 MPa was applied in dry-cured ham for 5 min, whereas our results showed a slight increase of this parameter immediately after HPP treatment at 450 MPa for 10 min at 12 °C. Such changes in shear strength have been shown to be attenuated during the storage. According to our results, enterocins A and B or thymol, individually or in combination with HPP, did not modify the shear force of sliced dry-cured ham.

Table 5 Texture characteristics (shear force, N) of sliced dry-cured ham treated with enterocins A and B (ENT, 1054 AU/g), thymol (THY, 1.25 mg/g), high pressure processing (HPP, 450 MPa/10 min), and their combinations during 30 days at 4 and 12 °C

		1 day	15 days	30 days
4 °C	Control	47.07 ± 8.17a	47.94 ± 15.10a	46.49 ± 7.56a
	ENT	42.81 ± 8.59a	47.11 ± 10.79a	42.98 ± 10.27a
	THY	41.77 ± 9.57a	39.27 ± 10.00a	45.17 ± 4.37a
	HPP	51.53 ± 12.54a	52.92 ± 15.95a	44.03 ± 10.65a
	ENT + HPP	39.97 ± 14.49a	40.38 ± 11.57a	43.33 ± 14.52a
	THY + HPP	42.65 ± 10.89a	43.63 ± 12.77a	43.86 ± 12.92a
12	Control	40.64 ± 11.65ab	41.76 ± 10.06a	40.21 ± 11.80a
	ENT	39.70 ± 13.83a	40.82 ± 12.45a	45.51 ± 14.45a
	THY	35.19 ± 8.65a	39.73 ± 6.68a	38.73 ± 11.05a
	HPP	53.89 ± 12.40b	51.14 ± 8.15a	47.12 ± 8.48a
	ENT + HPP	40.06 ± 15.90a	42.80 ± 11.77a	43.06 ± 12.77a
	THY + HPP	37.70 ± 7.04a	42.11 ± 9.58a	38.86 ± 12.86a

Values are the mean ± SD. Means within the same column with different lowercase letters differ significantly at $P < 0.05$ for a given temperature. Means within the same row with different uppercase letters differ significantly at $P < 0.05$

Conclusions

Enterocins A and B exhibited a strong antimicrobial effect against *L. monocytogenes* in dry-cured ham, whereas a regrowth was registered from day 15 onwards. HPP at 450 MPa for 10 min or thymol showed low antimicrobial activity against the pathogen. Combined treatment of enterocins A and B and HPP exhibited a synergist bactericidal effect during 30 days of storage at 4 and 12 °C and inhibited the growth of TVC, delaying the spoilage of dry-cured ham. Based on these findings, it can be concluded that combined treatment of enterocins A and B and HPP might be applied as a useful technology to avoid the growth of *L. monocytogenes* in case of contamination during the processing of sliced dry-cured ham and to extend the shelf life of the product with minor changes in its physicochemical, rheological, and color characteristics or induce consumer rejection.

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Capítulo 3

Efecto de los tratamientos de inactivación en la virulencia de *L. monocytogenes*

Los tratamientos como las APH y la bioconservación son eficaces en la inactivación de *L. monocytogenes* en jamón curado. Sin embargo, y a pesar de su efectividad, es muy difícil que eliminen toda la carga microbiana. La exposición a condiciones de estrés subletales puede derivar en la adaptación del patógeno a condiciones de estrés letales y/o producir cambios en su expresión génica, por lo que se consideró interesante investigar cómo afectan estos tratamientos a la fisiología o la capacidad de virulencia del patógeno en jamón curado. Así, el tercer objetivo de esta tesis fue estudiar la expresión génica de células de *L. monocytogenes* supervivientes a los tratamientos antimicrobianos, con el fin de evaluar los posibles cambios en su virulencia. En la primera parte del capítulo, se investigó el efecto de las altas presiones en la expresión de genes de virulencia y resistencia a estrés de *L. monocytogenes* inoculada en jamón curado loncheado. En la segunda parte, se estudió el efecto de la actividad de agua en la expresión de genes de virulencia y resistencia a estrés de *L. monocytogenes* en jamón curado presurizado. Finalmente, se investigó el efecto de un extracto de enterocinas en la expresión de genes de virulencia, principalmente relacionados con adhesión e invasión, de *L. monocytogenes* en jamón curado loncheado. Los resultados obtenidos en este capítulo se recogen en dos publicaciones científicas incluidas en el *Science Citation Index* (SCI) y una tercera enviada para publicación:

Publicación 3.1: **Pérez-Baltar, A.**, Serrano, A., Medina, M., & Montiel, R. (2021). Effect of high pressure processing on the inactivation and the relative gene transcription patterns of *Listeria monocytogenes* in dry-cured ham. *LWT - Food Science and Technology*, *139*, 110555. https://doi.org/10.1016/j.lwt.2020.110555

Publicación 3.2: **Pérez-Baltar, A.**, Alía, A., Rodríguez, A., Córdoba, J. J., Medina, M., & Montiel, R. (2020). Impact of water activity on the inactivation and gene expression of *Listeria monocytogenes* during refrigerated storage of pressurized dry-cured ham. *Foods*, *9*, 1092. https:// doi:10.3390/foods9081092

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Effect of high pressure processing on the inactivation and the relative gene transcription patterns of *Listeria monocytogenes* in dry-cured ham

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ABSTRACT

The population of *Listeria monocytogenes* and the expression of five virulence and stress-related genes in pressurized dry-cured ham were monitored throughout storage at 4 °C. *L. monocytogenes* strains S2 (serotype 1/2a) and S7-2 (serotype 4b) exhibited a moderate resistance to high pressure processing (HPP) at 450 MPa for 10 min or 600 MPa for 5 min. The low water activity (a_w) of sliced dry-cured ham ($a_w = 0.88$) diminished the efficacy of the treatments, with pathogen reductions lower than 1.5 log units. Regarding gene expression, a strain-dependent difference in gene transcription pattern was observed. All target genes (*prfA, plcA, hly, sigB* and *lmo1421*) were hardly downregulated for strain S2 immediately after pressurization, whereas were upregulated for strain S7-2. The initial overexpression exhibited by S7-2 was attenuated during the storage and after 30 d at 4 °C, virulence and stress-related genes were repressed, with fold changes up to 5.3, especially for 600 MPa treated samples.

1. Introduction

Listeria monocytogenes can cause listeriosis, a serious foodborne disease with the highest mortality rate in the European Union, which was 15.6% of confirmed cases in 2018 (EFSA & ECDC, 2019). Clinical listeriosis mainly occurs in vulnerable groups, such as the elderly, pregnant women, newborns, and immunocompromised patients and can lead to sepsis, meningitis, encephalitis, spontaneous abortion, or death (de Noordhout et al., 2014). *L. monocytogenes* is able to grow under stress conditions encountered in foods, such as refrigeration temperature (<5 °C), low pH (>4.3) and high levels of NaCl (up to 12%) (Lado & Yousef, 2007). Moreover, this pathogen can form biofilms on surfaces, as well as in pipes and drains of the food-processing environment, where is widely distributed, persisting for a long time (Crespo Tapia, den Besten, & Abee, 2018). *L. monocytogenes* can be found in a wide range of food products, including cheese, raw milk, vegetables, smoked fish or deli meats. Almost 99% of listeriosis cases are related to the consumption of contaminated foods, including RTE (ready-to-eat) meat products (Scallan et al., 2011).

Sliced dry-cured ham is a RTE meat product, which can be contaminated with *L. monocytogenes* during its industrial processing, mainly during the deboning, slicing and packaging or because of poor hygiene practices of handlers. Although *L. monocytogenes* is not able to grow in dry-cured ham throughout storage (Hereu, Bover-Cid, Garriga, & Aymerich, 2012), food processors have implemented strategies such as high pressure processing (HPP) (Aymerich, Picouet, & Monfort, 2008) to inactivate the pathogen in packaged and sliced dry-cured ham allowing its international commercialization. However, the limited effect of this treatment due to the baroprotection attributed to the low a_w values in dry-cured ham has been reported (Hereu et al., 2012; Serra-Castelló, Jofré, Garriga, & Bover-Cid, 2020).

L. monocytogenes adaptation to sub-lethal stresses results in changes in gene expression profiles (Ferreira, Wiedmann, Teixeira, & Stasiewicz, 2014). Presence of *sigB* gene contributed to the adaptation mechanisms of *L. monocytogenes* to HPP (Wemekamp-Kamphuis et al., 2004) and other environmental stresses such as acidic, osmotic or oxidative conditions, cold and nutrient limitation both outside and within the host (Dorey, Marinho, Piveteau, & O'Byrne, 2019). Moreover, SigB contributes to transcription of the *prfA* virulence gene cluster, the major virulence genetic locus identified in *L. monocytogenes* (Liu et al., 2019; Rantsiou, Mataragas, Alessandria, & Cocolin, (2012). The PrfA regulon is selectively activated during host cell infection and exerts a positive control on some factors of virulence, among which are included *plcA* and *hly*. The effect on the expression and the regulation of virulence and stress-related genes in HPP-surviving *L. monocytogenes* in food systems has rarely been studied.

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The aim of this work was to investigate the survival and the relative expression of genes involved in virulence and osmotic stress response of two strains of *L. monocytogenes* upon exposure to HPP in sliced dry-cured ham and during refrigerated storage.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Two *L. monocytogenes* strains (S2, serotype 1/2a and S7-2, serotype 4b) isolated from an Iberian pig processing plant were used as test organisms. Strain S2 was considered a persistent strain found in the environment, equipment, carcasses, and raw and dry-cured pork meat products, and strain S7-2 was non-persistent and isolated from raw products (Ortiz et al., 2010). The strains were maintained as stocked cultures at −80 °C in Brain Heart Infusion broth (BHI, Biolife s.r.l., Milano, Italy) supplemented with 20% glycerol. Before use in experiments, strains were sub-cultured twice in Tryptic Soy Yeast Extract Broth (TSYEB, Biolife) at 37 °C for 18 h.

2.2. Dry-cured ham preparation and HPP

One large piece (~7 kg) of deboned dry-cured ham was purchased from a commercial supplier in Spain and aseptically sliced in the laboratory. The chemical composition of ham was as follows: pH 5.7; a_w = 0.88; moisture content (%) 48.2; NaCl (%) 2.7; and nitrites content (mg/kg) 2.9. Ten g samples of dry-cured ham were inoculated by adding a cell suspension in 0.1% (wt/vol) peptone water solution (Biolife) of *L. monocytogenes* S2 or S7-2 on the surface of the sliced dry-cured ham to achieve a final concentration of ca. 10^7 CFU/g. Cell suspensions were prepared from overnight cultures in BHI broth, whose concentration was evaluated by plating on duplicate plates of CHROMagar *Listeria* (CH-L, Scharlab S.l., Barcelona, Spain). Dry-cured ham samples were individually vacuum-packaged in high-barrier multilayer bags (200 mm × 300 mm, BB325, Cryovac Sealed Air Corporation, Milan, Italy) and were pressurized at 450 MPa for 10 min or 600 MPa for 5 min. High pressure processing was carried out in a 135-Liter-capacity isostatic press (Hiperbaric, Burgos, Spain). The compression rate was approximately 200 MPa/min and the pressure release time was 3 s. The water temperature used as pressure-transmitting medium was 16 °C. Sliced dry-cured ham, inoculated with either of the two *L. monocytogenes* strains, but not pressurized, was used as control. After treatment, pressurized and non-pressurized samples were stored under refrigeration at 4 °C during 30 d. Three independent trials with duplicate samples were conducted.

2.3. L. monocytogenes enumeration

Samples of sliced dry-cured ham were analysed at 1, 7 and 30 d after pressurization to determine viable counts of *L. monocytogenes*. Ten g samples were transferred to stomacher bags, diluted 10-fold with sterile 0.1% (wt/vol) peptone water solution and homogenized for 120 s using a Silver Masticator homogenizer (IUL Instruments, Barcelona, Spain). Serial decimal dilutions of the homogenates were prepared in the same sterile solution. *L. monocytogenes* counts were determined on duplicate plates of CH-L, incubated at 37 °C for 48 h.

2.4. RNA extraction and cDNA synthesis

RNA extraction was performed at 0 and 6 h and 1, 7 and 30 d after pressurization by using the procedure described by Rantsiou, Mataragas, Alessandria, & Cocolin, (2012) with some modifications. Briefly, samples were diluted 10-fold with sterile 0.1% (wt/vol) peptone water solution and homogenized in a stomacher for 120 s. Four mL of the homogenates were centrifuged at 10,000×*g* for 5 min and 50 μL of RNA*later* (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) was added to the pellet. Fifty μL of lysozyme (50 mg/mL; Sigma-Aldrich) were then added to samples and were incubated at 37 °C for 20 min in a Thermomixer compact (Eppendorf Scientific, Hamburg, Germany). Total RNA was extracted using the MasterPure™ complete DNA and RNA purification kit (Epicentre, Madison, WI, USA) following the instructions of the manufacturer. Residual DNA was digested using the Turbo DNase (Invitrogen, Thermo Fisher Scientific, Walthman, MA, USA) and complete removal of the DNA was verified by quantitative PCR (qPCR) (as described in section 2.5) using an aliquot of the extract as template. DNase treatment was repeated until complete removal of the DNA. Then, RNA was quantified using the Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and normalized to 100 ng/μL. cDNA synthesis was performed using the GoScript™ Reverse Transcription Mix, Random Primers (Promega, Madison, WI, USA), according to the manufacturer's protocol. Approximately 2 μg of total RNA were used and retrotranscription (RT) reaction was carried out at 42 °C for 1 h. Reaction was stopped by inactivation of the enzyme at 70 °C for 15 min. The cDNA was stored at −20 °C until use.

2.5. Quantitative PCR

Quantitative PCR (qPCR) amplification was carried out for five representative virulence and stress-response genes (Table 1) previously employed in studies of *L. monocytogenes* gene expression *in situ* (Alía, Rodríguez, Andrade, Gómez, & Córdoba, 2019; Liu et al., 2019). Further, *IGS* was selected as a reference gene and internal control. Three biological replicates were analysed in a 96-well plate (VWR International, Radnor, PA, USA) for each gene of interest and each sample was amplified in duplicate. Two replicates of a DNA control sample together with a template-free negative control were also included in the runs. Plates were sealed with optical adhesive covers (Bio-Rad Laboratories, Hercules, CA, USA). In order to minimize the variance introduced by the instrument between the runs (inter-runs), all the samples were assayed for each gene separately in the same plate. The qPCR assays were carried out using the Mx3000P Real-Time PCR system (Agilent Technologies, Santa Clara, CA, USA), with the use of GoTaq® Probe qPCR Master Mix (Promega). Reactions (final volume of 25 μL) contained: 12.5 μL of the 2X GoTaq® Probe qPCR Master Mix, 0.9 mM *(prfA, lmo1421)* or 0.4 mM *(IGS, plcA, hly, sigB)* of each primer, 0.25 mM of the probe and 2 μL of cDNA template. The amplification program consisted of one cycle at 95 °C for 3 min, followed by 40 cycles of 15 s at 95 °C, 30 s at 50 °C (*plcA, hly*) or 1 min at 60 °C (*IGS, prfA, sigB, lmo1421*), and 30 s at 72 °C (*plcA, hly*). Previously, the PCR efficiency of each primer pair was determined using 10-fold dilutions of genomic DNA extracted from both *L. monocytogenes* strains as a template and adequate amplification efficiencies for target and reference genes were obtained.

2.6. Data and statistical analysis

Threshold cycle (C_T) values from qPCR assays were used for relative quantification. Mean C_T values for each analysis condition were obtained to calculate the relative gene transcription levels (fold changes) by the $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta C_T$ is: $(C_{T\text{target}} - C_{T\text{reference gene}})_{\text{test condition}} - (C_{T\text{target}} - C_{T\text{reference gene}})_{\text{control condition}}$ (Livak & Schmittgen, 2001). Stress or virulence genes were targets, while *IGS* was a reference gene, whose transcription is considered stable even under experimental treatments. The control condition was the non-pressurized sliced dry-cured ham inoculated with *L. monocytogenes*, while the test condition was the dry-cured ham inoculated with *L. monocytogenes* and pressurized at 450 MPa for 10 min or 600 MPa for 5 min at five different time points after treatments.

SPSS Statistics 22.0 software (IBM Corp., Armonk, NY, USA) was used to evaluate the significant differences between *L. monocytogenes* counts, as wells as to carry out the statistical treatment of $\log_2$ values of relative gene expression.

Table 1
Listeria monocytogenes genes targeted by qPCR in this study to determine the effect of HPP (450 MPa for 10 min and 600 MPa for 5 min) on virulence and stress gene expression.

Gene name	Function and scope of use	Sequence (5'→ 3')	Reference
IGS	Reference gene	IGS1: GGCCTATAGCTCAGCTGGTTA IGS2: GCTGAGCTAAGGCCCCATAAA P: HEX-CCATCGACCTCACGCTTATCAGGC-TAMRA	Rantsiou et al., (2012) This study
prfA	Transcriptional regulator, virulence	F: CAATGGGATCCACAAGAATATTGTAT R: AATAAAGCCAGACATTATAACGAAAGC P: HEX-TGTAAATTCATGATGGTCCCGTTCTCGCT-TAMRA	Kazmierczak, Wiedmann, & Boor, (2006)
plcA	Escape from primary vacuoles	F: CTAGAAGCAGGAATACGGTACA R: ATTGAGTAATCGTTTCTAAT P: HEX-AATTTATTTAAATGCATCACTTTCAGGT-TAMRA	Rantsiou et al., (2012)
hly	Lysis of vacuoles	F: TACATTAGTGGAAAGATGG R: ACATTCAAGCTATTATTTACA P: HEX-GAAAAATATGCTCAAGCTTATCCAAATG-TAMRA	Rantsiou et al., (2012)
sigB	Transcription factor, regulation of virulence and stress-response genes	F: CCAAGAAAATGGCGATCAAGAC R: CGTTGCATCATATCTTCTAATAGCT P: HEX-TGTTCATTACAAAAACCTAGTAGAGTCCAT-TAMRA	Rantsiou et al., (2012)
lmo1421	Glycine betaine ABC transporter, osmotic stress-related gene	F: CCACTGACAACTGGAACCATTTATA R: GAAAGAGCGCAATTTGTTGTAAAA P: HEX-ATGAACTTCGCTGGGATATCGGCT-TAMRA	Sue et al. (2003) This study

F, forward; R, reverse; P, probe; HEX, fluorochrome at 5′-end of the probe; TAMRA, quencher of HEX at 3′-end of the probe.

3. Results

3.1. Effect of HPP on L. monocytogenes population

L. monocytogenes counts in control and pressurized sliced dry-cured ham stored at 4 °C during 30 d are shown in Table 2. *L. monocytogenes* S2 and S7-2 initial counts in non-pressurized dry-cured ham were 7.3 and 7.4 log CFU/g, respectively. When dry-cured ham was treated at 450 MPa for 10 min, S2 and S7-2 levels were reduced by 0.8 log CFU/g. These reductions reached 1.3 and 1.5 log units, respectively, after 600 MPa for 5 min. *L. monocytogenes* S2 and S7-2 counts in control samples slightly declined by about 0.6 and 0.4 log units, respectively, throughout the storage, whereas remained unaltered in pressurized dry-cured ham at 450 and 600 MPa for up to 30 days at 4 °C.

3.2. Effect of HPP on L. monocytogenes gene expression

Figs. 1 and 2 show the relative gene transcription profiles of five virulence and stress-related genes (*prfA, plcA, hly, sigB* and *lmo1421*) induced by HPP during 30 d of storage at 4 °C for *L. monocytogenes* S2 and S7-2, respectively. As can be appreciated in figures, significant differences between the gene expression profiles of the two *L. monocytogenes* strains belonging to different serotype were observed. For strain S2, a slight downregulation for all target genes was observed immediately after HPP, but differences in gene expression between control and pressurized samples were not statistically significant. Contrary, for strain S7-2, a significant increase ($P < 0.05$) in the expression of *prfA, hly* and *sigB* genes was observed immediately after pressurization, with fold-changes higher than 8 for *hly* gene. Generally, such overexpression was higher in samples pressurized at 450 MPa than those treated at 600 MPa, being statistically significant ($P < 0.05$) for *prfA* gene.

The expression of the target genes fluctuated significantly ($P < 0.001$) during refrigeration time. Thus, for strain S2, with the exception of *sigB* gene, an overall upregulation trend was recorded throughout the storage. After 30 d at 4 °C, *hly* gene showed the highest relative expression levels, with fold changes values around 3, but such differences between test and control conditions were not statistically significant. In contrast, for strain S7-2, *prfA, hly, sigB* and *lmo1421* genes expression was significantly ($P < 0.01$) downregulated throughout the refrigeration. At day 30, all genes were repressed with respect to non-pressurized control condition, mainly *prfA* and *hly,* being such down-regulation higher in samples subjected to 600 MPa.

Table 2
Listeria monocytogenes S2 and S7-2 counts (log CFU/g) in sliced dry-cured ham pressurized at 450 MPa for 10 min or 600 MPa for 5 min during 30 d at 4 °C.

		Time (d)		
Strain	Treatment	1	7	30
S2	NP	7.0 ± 0.2bA	6.8 ± 0.3bA	6.7 ± 0.4bA
	450 MPa/10 min	6.5 ± 0.5bA	6.4 ± 0.6abA	6.3 ± 0.7abA
	600 MPa/5 min	6.0 ± 0.6 aA	5.9 ± 0.6 aA	6.0 ± 0.5 aA
S7-2	NP	7.2 ± 0.1 cB	7.1 ± 0.1 cB	7.0 ± 0.1 cA
	450 MPa/10 min	6.6 ± 0.4bA	6.6 ± 0.7bA	6.6 ± 0.3bA
	600 MPa/5 min	5.9 ± 0.5 aA	6.0 ± 0.6 aA	6.1 ± 0.2 aA

L. monocytogenes S2 and S7-2 initial counts (0 h) in control sliced dry-cured ham were 7.3 and 7.4 log CFU/g, respectively.
NP, non-pressurized.
Values are the mean ± SD. Means within the same column with different lowercase letters differ significantly at $P < 0.05$ for a given strain. Means within the same row with different uppercase letters differ significantly at $P < 0.05$.

4. Discussion

The efficacy of HPP inactivation depends on different factors, including the type of microorganism, its growth phase, the pressure applied, the time and temperature of processing, the composition of the food matrix and product characteristics such as pH and a_w (Campus, 2010). In this work, both strains of *L. monocytogenes* exhibited a similar and mild resistance to the pressure treatments applied, with reductions not higher than 1.5 log units. The HPP effect on *L. monocytogenes* viability has been widely investigated in RTE meat products, including dry-cured ham. Pérez-Baltar, Serrano, Bravo, Montiel, & Medina, (2019) studied the effect of HPP on the inactivation of a four-strain cocktail of *L. monocytogenes* in sliced dry-cured ham and found low reductions of only about 0.5 log units when 450 MPa for 10 min was applied. Similar results were observed in this work with *L. monocytogenes* S2 or S7-2. Jofré, Aymerich, Grèbol, & Garriga, (2009) found pathogen reductions of more than 3 log units in dry-cured ham treated at 600 MPa for 6 min and with a_w values of 0.918. Reductions on *L. monocytogenes* population attained in the present work at 600 MPa for 5 min were lower than 1.5 log units. The low a_w of sliced dry-cured ham ($a_w = 0.88$) could explain antimicrobial efficacy difference. Low a_w values protect microorganisms

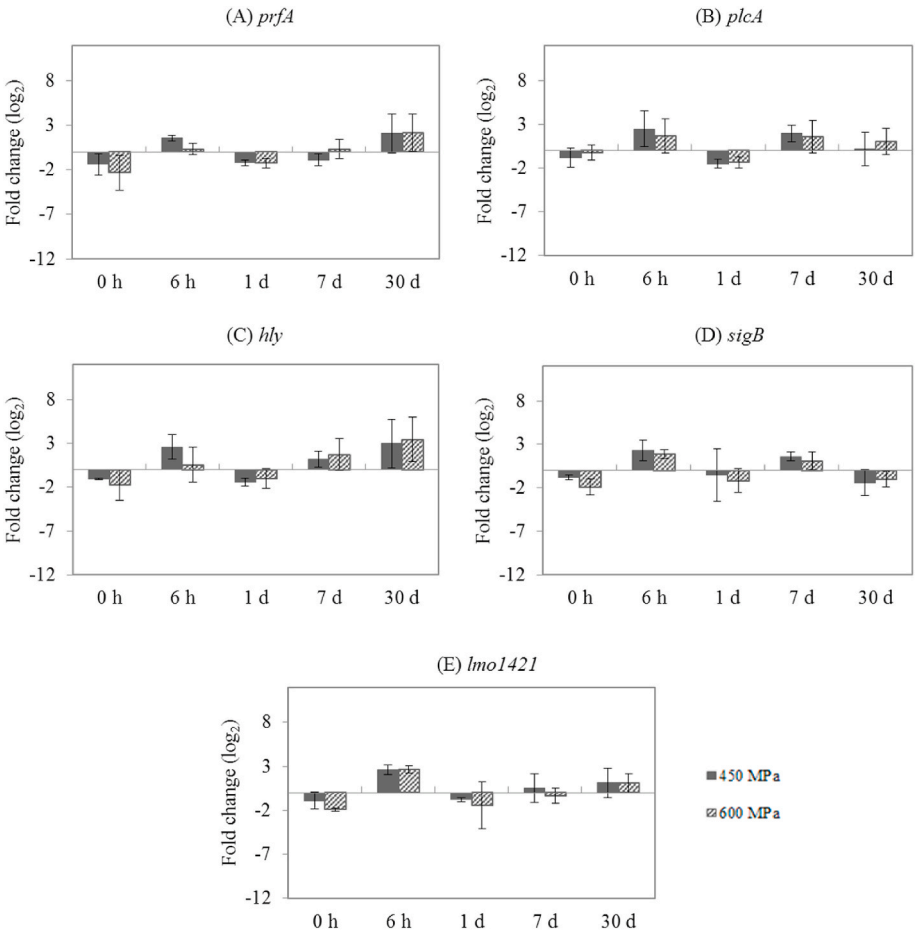

Fig. 1. Relative change in the transcription level for virulence and stress-related genes *prfA* (A), *plcA* (B), *hly* (C), *sigB* (D) and *lmo1421* (E) of *Listeria monocytogenes* strain S2 in sliced dry-cured ham pressurized at 450 MPa for 10 min or 600 MPa for 5 min during 30 d at 4 °C. Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method and $\log_2$ values are reported. Error bars indicate standard deviation of three biological replicates with duplicated samples (n = 6).

against high pressure and reduce the extent of bacterial inactivation induced by these treatments (Patterson, 2005). According to Morales, Calzada, & Nuñez (2016), the inactivation of *L. monocytogenes* in Iberian dry-cured ham (a_w = 0.90) by 450 MPa for 10 min was higher than in Serrano ham (a_w = 0.88). The baroprotective effect of low a_w on *L. monocytogenes* in dry-cured ham was also observed by Hereu et al. (2012) and Bover-Cid, Belletti, Aymerich, & Garriga, (2015) described the behaviour of *L. monocytogenes* in dry-cured ham under HPP as a function of water activity and fat content.

HPP is increasingly used in food industry to control *L. monocytogenes*, but how this non-thermal technology could modify the physiology of the pathogen in real food products has been barely studied. Therefore, the main purpose of this work was to evaluate the effect of HPP on the expression of five genes involved in virulence and stress response (*prfA, plcA, hly, sigB* and *lmo1421*) of *L. monocytogenes* survivors present in dry-cured ham after treatment and during storage. The results from the qPCR assays revealed that changes on HPP-surviving bacteria gene transcription patterns seemed to be strain-dependent. Broadly, strain S2 gene expression was slightly downregulated by pressurization, whereas strain S7-2 was significantly ($P < 0.05$) upregulated. A strain-dependent difference in gene expression profile was also observed when an enterocin AB extract or a bacteriocin-producing *Enterococcus faecalis* culture were used to control *L. monocytogenes* in dry-cured ham (Montiel et al., 2019) and after the exposure of the pathogen to salt (3.5%) in a cheese-based medium (Schrama, Helliwell, Neto, & Faleiro, 2013). Even strains belonging to the same serotype and subjected to acidic and NaCl stress have shown different behaviour regarding gene transcription and virulence potential (Olesen, Vogensen, & Jespersen, 2009).

A variety of stress resistance mechanisms that allow *L. monocytogenes* to adapt/survive to different environments encountered in foods have been reported. The alternative sigma factor, SigB (σ^B), and the genes under its control are known to contribute to osmoregulation (Sue, Boor, & Wiedmann, 2003), acid tolerance (Wemekamp-Kamphuis et al., 2004), bile tolerance (Zhang et al., 2011) or cell wall acting antimicrobials (Begley, Hill, & Ross, 2006). SigB also contributes to overcome food processing such as HPP (Wemekamp-Kamphuis et al., 2004) and plays an important role in virulence, inducing the transcriptional factor PrfA, the major regulator of the pathogen virulence. Bowman, Bittencourt, & Ross, (2008) reported the suppression of gene expression associated with cellular growth and virulence when HPP was applied on TSYE broth inoculated with *L. monocytogenes*. Thus, pressure treatments at 400 or 600 MPa for 5 min caused a downregulation of *sigB* and *prfA* genes for a *L. monocytogenes* strain belonging to serotype 1/2a. Such repression was greater at lower pressure (400 MPa). A suppression of *sigB* and *prfA* genes was also observed in this work immediately after applying 450 or 600 MPa for the strain S2. On the contrary, relative expression of both genes was significantly ($P < 0.05$) upregulated for S7-2 strain, being such induction more pronounced at 450 MPa. The initial overexpression registered for *prfA* and *sigB*, genes related with virulence and stress resistance of strain S7-2, was attenuated during the storage at 4 °C, recording a significant ($P < 0.01$) downregulation from 7 days onwards.

The PrfA-dependent expression is regulated by PrfA concentration as well as its affinity for the promoter. The *plcA* and *hly* promoters have a perfectly symmetrical, high-affinity PrfA box and are considerably sensitive to this regulator (de las Heras, Cain, Bielecka, & Vázquez-Boland,

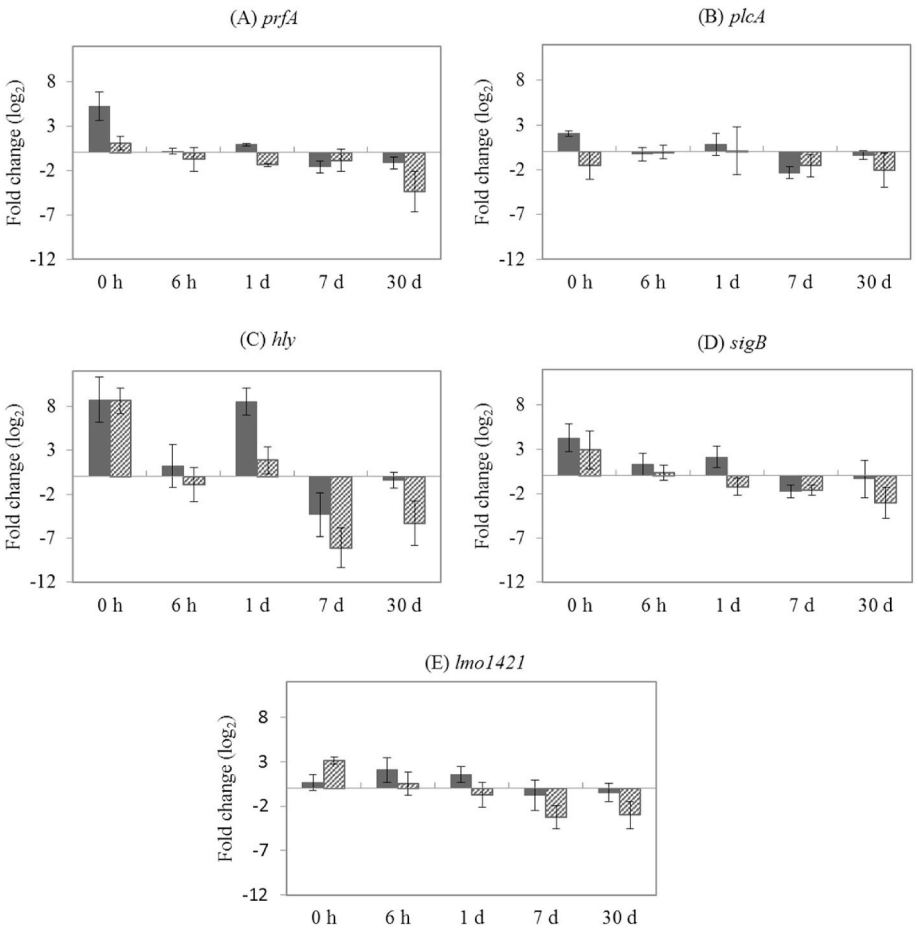

Fig. 2. Relative change in the transcription level for virulence and stress-related genes *prfA* (A), *plcA* (B), *hly* (C), *sigB* (D) and *lmo1421* (E) of *Listeria monocytogenes* strain S7-2 in sliced dry-cured ham pressurized at 450 MPa for 10 min or 600 MPa for 5 min during 30 d at 4 °C. Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method and $\log_2$ values are reported. Error bars indicate standard deviation of three biological replicates with duplicated samples (n = 6).

2011). Thus, a positive regulation of *prfA* gene would result in a higher concentration of the PrfA factor and, consequently, in a higher activation of the *plcA* and *hly* genes. This fact could explain the trend of expression of these genes, which was similar to that observed for *prfA* gene. Immediately after pressure treatments, *hly* gene was slightly repressed for S2 strain and overexpressed for S7-2. Same tendency was registered for *plcA* gene, although the levels of transcription were lower than those observed for *hly*. It is known that *hly* gene has PrfA-independent promoters (Domann et al., 1993) which may contribute to the differential expression for *plcA* and *hly* genes, despite being both of them controlled by PrfA.

Physicochemical properties encountered in sliced dry-cured ham such NaCl content or a_w could also influence the gene transcription patterns of *L. monocytogenes* strains. NaCl concentrations common in RTE meat products increased the activity of the alternative sigma factor and induced an overexpression of *lmo1421,* a SigB-dependent gene related with general stress-response (Liu, Miller, Basu, & Mcmullen, 2014; Mataragas et al., 2015; Olesen, Thorsen, & Jespersen, 2010; Sue et al., 2003). However, in this work, pressure treatments at 450 and 600 MPa weakly affected the transcription profile of *lmo1421* for both strains of *L. monocytogenes* in dry-cured ham. Changes in gene expression (fold changes) was normalized to an endogenous reference gene and relative to non-pressurized dry-cured ham as a control condition. Therefore, the sole impact of HPP was observed while the stressful conditions encountered in dry-cured ham (NaCl, low a_w, refrigeration temperature and nitrites) that are known to have influence on gene expression are leveled out.

This work contributes new data about the impact of HPP on the expression of genes involved in virulence and stress response of *L. monocytogenes* in dry-cured ham. *L. monocytogenes* S2 (persistent) and S7-2 (non-persistent) artificially inoculated in dry-cured ham exhibited a similar resistance against HPP, but a different pattern regarding the expression of target genes could be delineated for both strains after HPP. Whereas *L. monocytogenes* S2 (serotype 1/2a) was hardly affected by pressure treatments, HPP-surviving cells of strain S7-2 (serotype 4b) exhibited a significant increase ($P < 0.05$) in the expression of *prfA, hly* and *sigB* genes. Briefly, although gene expression of *L. monocytogenes* S7-2 was initially induced by HPP, the surviving cells were not potentially more virulent or stress-resistant from day 7 onwards.

5. Conclusions

L. monocytogenes strains S2 and S7-2 exhibited a mild resistance to HPP (450 for 10 min and 600 MPa for 5 min) at low a_w sliced dry-cured ham. HPP treatments affected the expression of five virulence and stress-related genes (*prfA, plcA, hly, sigB* and *lmo1421*) but the two strains of the pathogen responded differently. More strains should be evaluated to elucidate if cellular response induced by HPP is associated with serotype or with other strain characteristics. This study highlights that gene expression may be influenced by HPP but surviving *L. monocytogenes* cells were not potentially more virulent or stress-resistant.

CRediT authorship contribution statement

Aida Pérez-Baltar: Data curation, Formal analysis, Investigation, Methodology, Software, Writing - original draft, Writing - review &

editing. **Alejandro Serrano:** Data curation, Methodology. **Margarita Medina:** Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing - review & editing. **Raquel Montiel:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that there is not conflict of interest regarding the publication "Effect of high pressure processing on the inactivation and the relative gene transcription patterns of *Listeria monocytogenes* in dry-cured ham" by Aida Pérez-Baltar et al.

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Publicación 3.2

foods

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Article

Impact of Water Activity on the Inactivation and Gene Expression of *Listeria monocytogenes* during Refrigerated Storage of Pressurized Dry-Cured Ham

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Abstract: *Listeria monocytogenes* population and the expression patterns of three virulence (*plcA*, *hly*, and *iap*) and one stress-related (*sigB*) genes in dry-cured ham with different water activity (a_w) values (0.92, 0.88, and 0.84) and treated with high pressure processing (HPP, 450 MPa/10 min and 600 MPa/5 min) were monitored throughout 30 days (d) at 4 °C. The antimicrobial effect of HPP at 600 MPa against *L. monocytogenes* S4-2 (serotype 1/2b) and S12-1 (serotype 1/2c) was greater in dry-cured ham with a_w values of 0.92, with reductions of 2.5 and 2.8 log units, respectively. The efficacy of HPP treatments decreased at lower a_w values. Regarding gene expression, *L. monocytogenes* strains responded differently to HPP. For strain S4-2, the four target genes were generally overexpressed in dry-cured ham immediately after HPP treatments at the three a_w values investigated, although the extent of this induction was lower in the samples pressurized at 600 MPa and with a_w values of 0.84. For strain S12-1, the expression of all target genes was repressed at the three a_w values investigated. The antimicrobial efficacy of HPP against *L. monocytogenes* could be compromised by low a_w values in food products. However, no growth of HPP-survival cells was observed during refrigerated storage in low-a_w dry-cured ham, and the overexpression of virulence and stress-related genes decreased.

Keywords: *L. monocytogenes*; high pressure processing; cured meat; qPCR; virulence and stress-related genes

1. Introduction

Listeria monocytogenes is a foodborne pathogen causing listeriosis, mainly in neonates, the elderly, and pregnant or immunocompromised individuals. Although listeriosis has low morbidity, it is the most severe human disease in terms of hospitalization (more than 90% of cases) and has the highest mortality rate in the European Union, which was 15.6% in 2018 [1]. Certain strains of *L. monocytogenes* can form biofilms and withstand cleaning and disinfection processes allowing the pathogen to persist in the food processing installations for long periods. Moreover, this pathogen is able to overcome environmental stresses encountered in foods, such as refrigeration temperature (<5 °C), low pH (>4), or high content of NaCl (up to 12%). However, it is inhibited at water activity (a_w) values below 0.92 [2].

Dry-cured ham can result contaminated with *L. monocytogenes* during post-processing steps (e.g., deboning or slicing). In the European Union, a maximum of 100 CFU/g of *L. monocytogenes* is allowed during the shelf-life for ready-to-eat (RTE) foods different to those intended for infants and medical

purposes and that do not support the growth of the pathogen [3]. A "zero tolerance" criterion (absence in 25 g) is required in the USA [4], which is an impediment for the international commerce of cured meat products. The establishment of strategies to control *L. monocytogenes* (e.g., biopreservation and/or high pressure processing, HPP) would boost the international market of dry-cured ham.

HPP is a non-thermal technology effective to control foodborne pathogens and spoilage microorganisms, and its industrial implementation is continually growing. HPP can inactivate microorganisms subjecting the products to elevated pressures [5], controlling pathogens such as *L. monocytogenes* in dry-cured ham with minor changes in texture, color, or nutritional value [6]. HPP efficacy depends on various processing parameters such as the pressure level, temperature, and time of processing, as well as the type of microorganism and its growth phase. Pathogen lethality during HPP also depends very highly on the composition and the physico-chemical properties of the food matrix such as pH and a_w [7–9]. Low a_w values could reduce the antimicrobial efficacy of HPP against *L. monocytogenes* in sliced dry-cured ham and could result in changes in virulence and stress-related gene expression profiles of surviving *L. monocytogenes* cells [10,11].

Stress-related gene *sigB* contributes to overcome different conditions encountered in foods such as acidic, osmotic, or oxidative stresses [12] and to the adaptation of the pathogen to HPP [13]. Moreover, it contributes to the transcription of the *prfA* virulence gene cluster, the major virulence genetic locus identified in *L. monocytogenes* [14], which exerts a positive control on some virulence factors, including *plcA* and *hly* genes. Studies considering the effect of dry-cured ham a_w on the inactivation of *L. monocytogenes* by HPP are scarce and the consequences of the treatments for the physiology of the pathogen do not usually be considered. Current knowledge regarding the antilisterial effect of HPP in dry-cured ham should be integrated with information concerning molecular or cellular response of the pathogen to such treatments. Changes in gene expression may be of great help to understand changes in microbial physiology [15].

The aim of this work was to investigate the effect of dry-cured ham water activity on the inactivation and the relative expression of genes involved in virulence and stress response of two strains of *L. monocytogenes* during refrigerated storage after HPP treatment.

2. Materials and Methods

2.1. Microorganisms

Two *L. monocytogenes* strains (S4-2—serotype 1/2b and S12-1—serotype 1/2c) from the environment of a pork processing industry [16] were used as target organisms. Stock cultures in brain heart infusion (BHI, Biolife S.r.l., Milano, Italy) at −80 °C were subcultured twice onto Tryptic Soy Yeast Extract Broth (TSYEB, Biolife) at 37 °C for 18 h before use in experiments.

2.2. Adjustment of a_w Values in Sliced-Dry Cured Ham

One deboned dry-cured ham was obtained from a local supplier in Spain and aseptically sliced in a laminar flow cabinet (Bio II Advance, Telstar, Terrassa, Spain). The slices were then separately placed in pre-sterilized receptacles with saturated chloride solution in the bottom, where the relative humidity was maintained constant following the procedure previously reported by Andrade et al. [17]. In these receptacles, the slices of ham were kept during 10 h and a_w values were determined every hour by means of an Aqualab Series 3 instrument (Decagon Devices Inc., Pullman, WA, USA) at room temperature. Slices of three different a_w values were achieved in the receptacles: 0.92, 0.88, and 0.84, simulating hams of different aging times (drying, cellar, and finished product, respectively). Two independent trials with triplicate samples were performed.

2.3. Dry-Cured Ham Inoculation and HPP Treatments

After a_w adjustment, slices of 10 g were inoculated by spreading 100 µL of a cell suspension of *L. monocytogenes* S4-2 or S12-1 in order to achieve a final population of approximately 10^6 CFU/g.

Cell suspensions were prepared from overnight cultures in BHI broth. Ten gram non-inoculated slices were used to determine total viable counts (TVC) and pH. Inoculated and non-inoculated dry-cured ham slices were individually vacuum-packaged in high-barrier multilayer bags (BB325, Cryovac Sealed Air Corporation, Milan, Italy) and kept at 4 °C for 20 h. Samples were treated at 450 MPa for 10 min or 600 MPa for 5 min in a prototype Wave 6000/135 (NC Hyperbaric, Burgos, Spain). The compression rate was 218 MPa/min and the pressure release time was 6 s. The temperature of water used as pressure-transmitting medium was 19 °C. Sliced dry-cured ham, inoculated with either of the two *L. monocytogenes* strains, but not pressurized, was used as control. After treatments, all samples were kept at 4 °C during 30 days (d). Three independent experiments with duplicate samples were conducted.

2.4. Microbiological Analysis

Inoculated and non-inoculated samples were analyzed at 1, 15, and 30 d after HPP. Ten gram samples were 10-fold diluted with sterile 0.1% (*wt/vol*) peptone water solution and homogenized for 120 s in a stomacher (IUL Instruments, Barcelona, Spain). Enumeration of *L. monocytogenes* in inoculated samples was performed on duplicate plates of CHROMagar *Listeria* (CH-L, Scharlab S.l., Barcelona, Spain) incubated at 37 °C for 48 h. TVC in non-inoculated samples were enumerated in duplicate on Tryptic Soy Agar (TSA, Panreac Química SLU, Barcelona, Spain) incubated at 30 °C for 72 h.

2.5. Physicochemical Analysis

Non-inoculated samples were used to determine pH, sodium chloride (NaCl), and nitrites (NO^{2-}) content of dry-cured ham. NaCl and NO^{2-} content were analyzed after the adjustment of a_w in non-pressurized dry-cured ham. pH determination was performed 1, 15, and 30 d after HPP in control and pressurized samples. Samples (5 g) were homogenized with 15 mL of distilled water for 120 s and pH was determined by means of a pH-meter GLP22 (Crison Instruments S.A., Barcelona, Spain). The reference method from ISO 2918:1975 [18] was used to evaluate nitrites content. The salt content was estimated as chloride using QUANTAB Chloride Titrator (Hach Company, Loveland, CO, USA) according to the Association of Official Analytical Chemists (AOAC) [19]. All analyses were performed in triplicate.

2.6. RNA Extraction and cDNA Synthesis

RNA extraction was performed at 0 h and 30 d after HPP. Samples were prepared as indicated in Section 2.4. One milliliter of the homogenates was used to extract total RNA using the MasterPure™ complete DNA and RNA purification kit (Epicentre, Madison, WI, USA) following the instructions of the manufacturer. A DNase treatment (Thermo Fisher Scientific, Walthman, MA, USA) was done to digest residual genomic DNA. Then, RNA concentration and purity were determined using a Nanodrop™ (Thermo Fisher Scientific) and normalized to 100 ng/µL. Reverse transcription reaction for cDNA synthesis was conducted in a Mastercycler® EP (Eppendorf Scientific, Hamburg, Germany) using the PrimeScript™ Reagent (Perfect Real Time) kit (TaKaRa Bio Inc., Dalian, China). Approximately, 5 µg of total RNA were retrotranscripted at 37 °C for 15 min. The reaction was stopped by inactivation of the enzyme at 85 °C for 5 s. Finally, cDNA was kept at −20 °C until use.

2.7. Quantitative PCR

Quantitative PCR (qPCR) based on TaqMan® methodology was used to amplify the virulence-associated genes *plcA*, *hly*, and *iap*, and stress-related gene *sigB* of *L. monocytogenes* (Table 1) as previously described Alía et al. [10]. *IGS* was selected as a reference gene and internal control. Two biological replicates were analyzed for each gene of interest and each sample was amplified in triplicate in MicroAmp Fast Optical 96-Well Reaction Plate (0.1 mL) (Thermo Fisher Scientific, Walthman, MA, USA). qPCR was carried out using the qPCR Vii™ 7 System (Thermo Fisher Scientific,

Walthman, MA, USA). Reactions (final volume of 12.5 µL) contained: 2.5 µL of cDNA templates, 7 µL of Premix ExTaq™ (TaKaRa Bio Inc., Dalian, China), 0.125 µL of 50x ROX™ Reference Dye (TaKaRa Bio Inc., Dalian, China), 300 nM (*IGS, plcA*, *iap*, and *sigB*), or 450 nM (*hly*) of each primer, and 100 nM (*IGS, hly*), 200 nM (*plcA*), or 300 nM (*iap* and *sigB*) of the probe. The amplification program consisted of one cycle at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C, 1 min at 55 °C (*IGS* and *plcA*), or 60 °C (*hly, iap,* and *sigB*).

2.8. Data and Statistical Analysis

Threshold cycle (C_T) values from qPCR were used for relative quantification. Mean C_T values for each analysis condition were obtained to calculate the relative gene transcription levels (fold changes) by the $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta C_T$ is $(C_{T\text{target}} - C_{T\text{reference gene}})_{\text{test condition}} - (C_{T\text{target}} - C_{T\text{reference gene}})_{\text{control condition}}$ [21]. Stress or virulence genes were considered targets, while *IGS* was considered a reference gene whose transcription is considered stable even under experimental treatments. Control condition corresponded to non-pressurized dry-cured ham, while test condition corresponded to pressurized dry-cured ham, at the different a_w values and the respective time points.

SPSS Statistics 22.0 software (IBM Corp., Armonk, NY, USA) was used to carry out the statistical treatment of $\log_2$ values of relative gene expression as well as to evaluate the significant differences between *L. monocytogenes* counts, TVC, and pH values. The Tukey test was applied to detect significant differences between means at $\alpha = 0.05$ [22].

Table 1. *L. monocytogenes* genes targeted by qPCR in this study.

Gene	Function and Scope of Use	Sequence (5′→3′)	Reference
IGS	Reference gene	F: GGCCTATAGCTCAGCTGGTTA	Rantsiou et al. 2012 [14]
		R: GCTGAGCTAAGGCCCCATAAA	
		P: FAM-ATAAGAAATACAAATAATCATACCCTTTTAC-TAMRA	
plcA	Escape from primary vacuoles	F: CTAGAAGCAGGAATACGGTACA	Rantsiou et al., 2012 [14]
		R: ATTGAGTAATCGTTTCTAAT	
		P: HEX-AATTTATTTAAATGCATCACTTTCAGGT-TAMRA	
hly	Lysis of vacuoles	F: CATGGCACCACCAGCATCT	Rodríguez-Lázaro et al., 2004 [20]
		R: ATCCGCGTGTTTCTTTTCGA	
		P: HEX-CGCCTGCAAGTCCTAAGACGCCA-TAMRA	
iap	Invasion associated secreted endopeptidase	F: AATCTTTAGCGCAACTTGGTTAA	Rodríguez-Lázaro et al., 2004 [20]
		R: CACCTTTGATGGACGTAATAATACTGTT	
		P: HEX-CAACACCAGCGCCACTAGGACG-TAMRA	
sigB	Transcription factor, regulation of virulence and stress-response genes	F: CCAAGAAAATGGCGATCAAGAC	Rantsiou et al., 2012 [14]
		R: CGTTGCATCATATCTTCTAATAGCT	
		P: HEX-TGTTCATTACAAAAACCTAGTAGAGTCCAT-TAMRA	

F, forward; R, reverse; P, probe; FAM/HEX, fluorochrome at 5′-end of the probe; and TAMRA, quencher of FAM/HEX at 3′-end of the probe.

3. Results

3.1. Adjustment of a_w in Sliced Dry-Cured Ham

Figure 1 shows the decrease of a_w values in sliced dry-cured ham over time. The initial a_w was 0.935 ± 0.004. Slices of dry-cured ham reached a_w values of 0.92, 0.88, and 0.84 after 1, 6, and 10 h exposed to saturated chloride solution, respectively. NaCl and NO^{2-} content of dry-cured ham after adjustment of a_w are indicated in Table 2. No statistical differences in NaCl and NO^{2-} content were detected as consequence of a_w modification. NaCl and NO^{2-} values ranged from 3.6 to 5.3% and from 3.8 to 4.3 mg/kg, respectively.

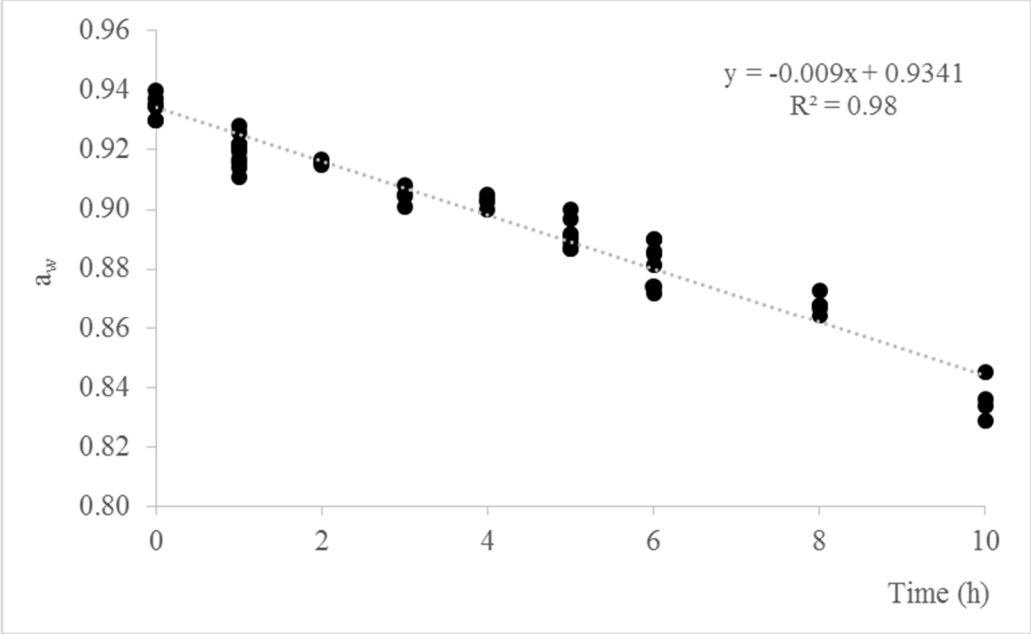

Figure 1. Adjustment of a_w values in sliced dry-cured ham after exposition to saturated chloride solution in sterilized receptacles during 10 h.

Table 2. Physicochemical characteristics of sliced dry-cured ham with different a_w values.

a_w	NaCl (% *wt/wt*)	Nitrites (mg/kg)
0.92	3.6 ± 0.3 a	3.9 ± 0.3 a
0.88	4.3 ± 1.3 a	3.8 ± 0.0 a
0.84	5.3 ± 0.0 a	4.3 ± 0.2 a

Values are means ± SD. Values in the same column with different lowercase indicate significant differences at $p \leq 0.05$.

3.2. Effect on L. monocytogenes Population

Reductions on *L. monocytogenes* S4-2 and S12-1 population in pressurized dry-cured ham with different a_w values during refrigerated storage at 4 °C are shown in Figures 2 and 3, respectively. One day after HPP, strain S4-2 levels were 6.6, 6.1, and 6.1 log CFU/g in non-pressurized dry-cured ham with 0.92, 0.88, and 0.84 a_w values, respectively. Reductions of *L. monocytogenes* by pressurization at 450 MPa were lower than 1.0 log in dry-cured ham with different a_w values. The inactivation of *L. monocytogenes* S4-2 was significantly ($p \leq 0.001$) higher at 600 MPa/5 min, with reductions of 2.5 log units in samples with the highest (0.92) a_w value. The antimicrobial activity of such pressure treatment decreased ($p \leq 0.05$) when it was applied in dry-cured ham with lower (0.88 and 0.84) a_w values, with levels 1.0 log units lower than in non-pressurized samples, respectively.

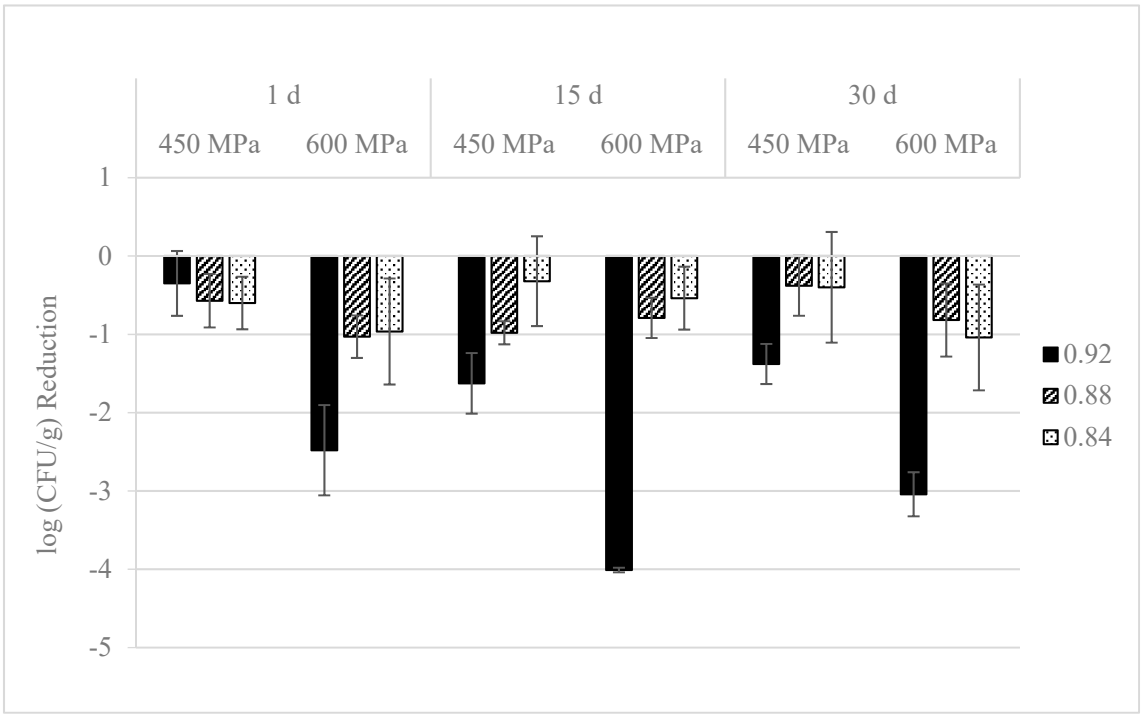

Figure 2. *L. monocytogenes* S4-2 inactivation (log CFU/g reduction) in sliced dry-cured ham with different a_w values (0.92, 0.88, and 0.84) treated with HPP (450 MPa/10 min or 600 MPa/5 min) and stored during 30 days at 4 °C. All values represent the average of three independent experiments with duplicate samples. Error bars designate standard deviation.

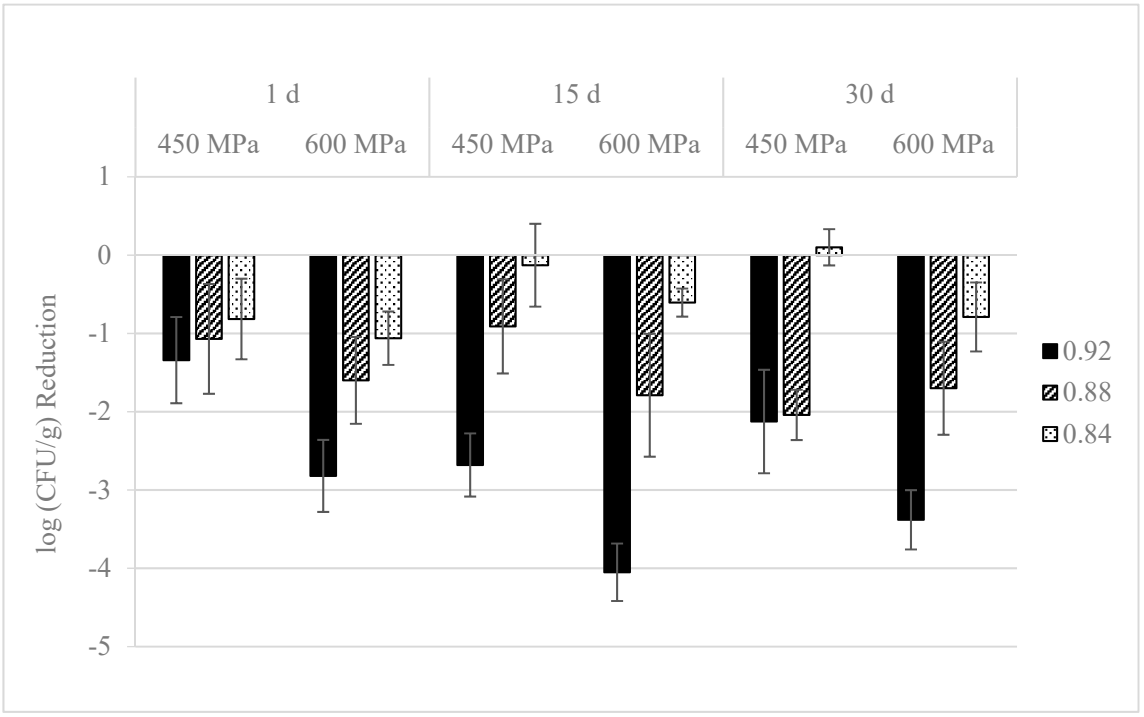

Figure 3. *L. monocytogenes* S12-1 inactivation (log CFU/g reduction) in sliced dry-cured ham with different a_w values (0.92, 0.88, and 0.84) treated with HPP (450 MPa/10 min or 600 MPa/5 min) and stored during 30 days at 4 °C. All values represent the average of three independent experiments with duplicate samples. Error bars designate standard deviation.

Regarding *L. monocytogenes* S12-1, initial counts were 6.5, 6.1, and 5.8 log CFU/g in control dry-cured ham with a_w values of 0.92, 0.88, and 0.84, respectively. Reductions lower than 1.5 log units were achieved when 450 MPa/10 min were applied in dry-cured ham with different a_w. HPP at 600 MPa/5 min resulted in higher reductions ($p \leq 0.001$) in the samples with the highest (0.92) a_w value, with counts 2.8 log units lower than those observed in non-pressurized dry-cured ham. The antilisterial effect of such treatment diminished ($p \leq 0.05$) when it was applied in samples with lower (0.88 and 0.84) a_w, with levels 1.8 and 1.1 log CFU/g lower than those in non-pressurized ham, respectively.

During refrigeration storage, *L. monocytogenes* S4-2 and S12-1 counts kept stable in control (non-pressurized) 0.92 a_w samples, with counts after 30 d approximately 0.2 log units lower than those observed at day 1. Pathogen counts decreased slightly during storage in dry-cured ham with 0.88 and 0.84 a_w values, with differences between strains. The reduction was 0.5 log units after 30 d and a_w values of 0.88 and 0.84 for strain S4-2, and 0.3 and 1.0 log units at 0.88 and 0.84, respectively, for strain S12-1.

Strain S4-2 levels significantly ($p \leq 0.05$) diminished throughout storage in samples pressurized at 450 and 600 MPa and a_w of 0.92, with counts 1.4 and 3.0 log units lower, respectively, than those observed in control ham at day 30, whereas kept invariable in samples with lower (0.88 and 0.84) a_w. Concerning strain S12-1, levels also diminished ($p \leq 0.05$) throughout the refrigerated storage of cured ham with a_w of 0.92 and pressurized at 450 and 600 MPa. Counts were 2.1 and 3.4 log CFU/g lower than those observed in non-pressurized control ham at day 30, respectively. In samples with a_w of 0.88, these reductions were 2.0 and 1.7 log units, respectively, whereas in dry-cured ham with a_w of 0.84, pathogen levels did not change during the storage.

3.3. Effect on TVC and pH

Reductions on TVC in control and pressurized dry-cured ham with different a_w values during storage at 4 °C are represented in Figure 4. At day 1, TVC in control samples were 5.4, 5.7, and 6.1 log CFU/g in dry-cured ham with 0.92, 0.88, and 0.84 a_w values, respectively. HPP significantly ($p \leq 0.05$) diminished microbial levels in dry-cured ham with different a_w values. Reductions of TVC by 450 MPa/10 min were 1.1, 1.7, and 1.1 log units in samples with a_w values of 0.92, 0.88, and 0.84, respectively. Such reductions by 600 MPa/5 min were 1.3, 1.9, and 1.1 log CFU/g, respectively. During refrigerated storage, TVC slightly increased ($p \leq 0.05$) in non-pressurized dry-cured ham with a_w values of 0.92, but this growth was avoided by HPP. TVC population kept unchanged in pressurized and non-pressurized samples with 0.88 and 0.84 a_w values throughout 30 d of storage.

Values of pH in control and pressurized dry-cured ham with different a_w values during storage at 4 °C are indicated in Table 3. One day after HPP, pH values ranged from 5.82 to 6.01 in control and pressurized dry-cured ham with 0.92–0.84 a_w values. There were no significant differences in pH values when pressure was applied in dry-cured ham with different a_w values. During refrigeration, values of pH kept constant in control and treated dry-cured ham with higher a_w values, whereas a slight decrease ($p \leq 0.05$) was registered in samples with a_w of 0.88 and 0.84, with values not lower than 5.78 after 30 d at 4 °C.

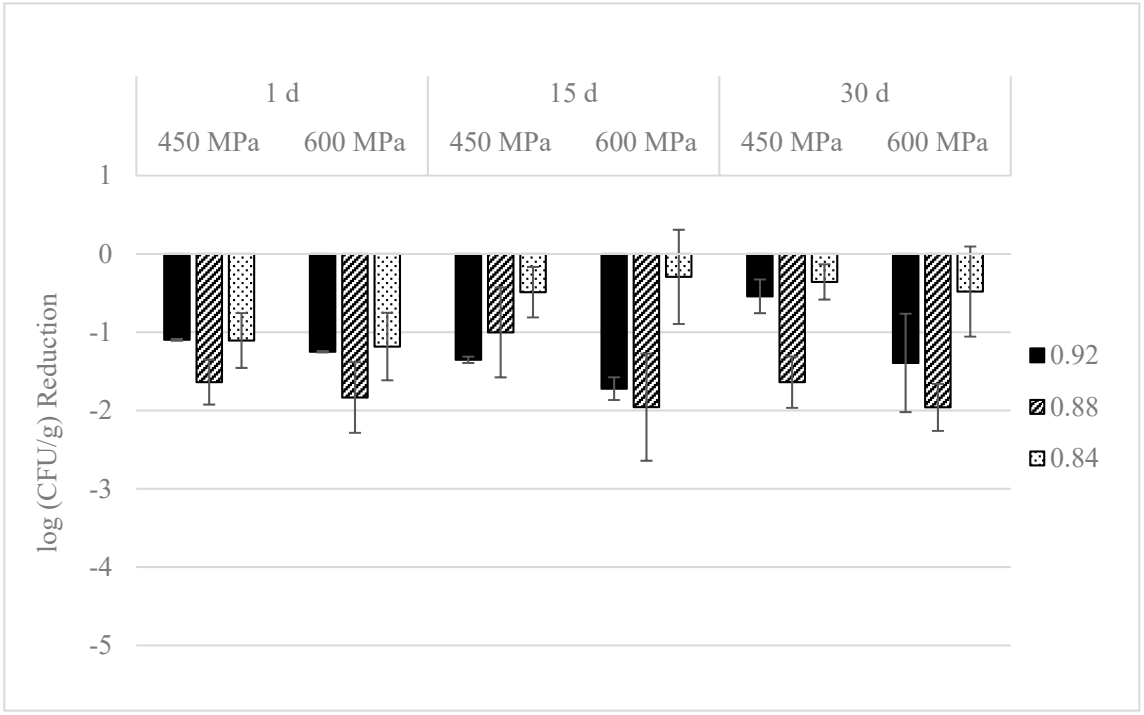

Figure 4. Total viable counts (TVC, log CFU/g reduction) in sliced dry-cured ham with different a_w values (0.92, 0.88, and 0.84) treated with HPP (450 MPa/10 min or 600 MPa/5 min) and stored during 30 days at 4 °C. All values represent the average of three independent trials with duplicate samples. Error bars designate standard deviation.

Table 3. Values of pH in sliced dry-cured ham with different a_w values (0.92, 0.88, and 0.84) and treated with HPP (450 MPa/10 min or 600 MPa/5 min) during 30 days at 4 °C.

a_w	Treatment	Time (d)		
		1	15	30
0.92	NP	5.82 ± 0.04 aA	5.82 ± 0.08 aA	5.89 ± 0.07 aA
	450 MPa/10 min	5.88 ± 0.12 aA	5.91 ± 0.08 aA	5.87 ± 0.07 aA
	600 MPa/5 min	5.95 ± 0.08 aA	6.06 ± 0.07 bB	5.85 ± 0.02 aA
0.88	NP	5.88 ± 0.07 aB	6.07 ± 0.06 bC	5.78 ± 0.06 aA
	450 MPa/10 min	5.86 ± 0.13 aAB	5.98 ± 0.05 aB	5.80 ± 0.05 aA
	600 MPa/5 min	5.92 ± 0.02 aB	5.96 ± 0.04 aB	5.86 ± 0.06 aA
0.84	NP	5.93 ± 0.08 aA	6.12 ± 0.05 aB	5.86 ± 0.03 abA
	450 MPa/10 min	6.01 ± 0.12 aA	6.12 ± 0.02 aB	5.91 ± 0.03 bA
	600 MPa/5 min	5.97 ± 0.07 aB	6.14 ± 0.12 aC	5.83 ± 0.05 aA

Values are means ± SD. NP, non-pressurized. Means within the same column with different lowercase indicate significant differences at $p \leq 0.05$ for a given a_w. Values in the same row with different uppercase indicate significant differences at $p \leq 0.05$.

3.4. Effect on L. monocytogenes Gene Expression

The relative gene transcription profiles of four virulence and stress-related genes (*plcA*, *hly*, *iap*, and *sigB*) of *L. monocytogenes* S4-2 and S12-1 in sliced dry-cured ham with different a_w (0.92, 0.88, and 0.84) and treated with HPP (450 MPa/10 min or 600 MPa/5 min) during 30 d at 4 °C are shown in Figures 5 and 6, respectively. Relative gene expression profiles of the two *L. monocytogenes* strains belonging to different serotype were significantly different. For strain S4-2 (serotype 1/2b), an overall

upregulation was registered in dry-cured ham at the three a_w values investigated, immediately after HPP, which was more noticeable for *hly* and *sigB* genes. Generally, such overexpression was higher in samples with higher a_w values and pressurized at 450 MPa/10 min, being statistically significant ($p \leq 0.05$) for *sigB* and *iap* genes. Contrary, for strain S12-1 (serotype 1/2c), all target genes were significantly repressed ($p \leq 0.01$) immediately after pressurization, being more pronounced for *hly* and *plcA* genes. This downregulation was greater in pressurized dry-cured ham with 0.92 and 0.88 a_w values, with fold changes higher than nine for *hly* gene.

The expression of the target genes fluctuated during refrigeration storage. For strain S4-2, the overexpression initially recorded for all genes was reduced during the 30 d of the refrigerated storage, which was generally lower in dry-cured ham with 0.88 and 0.84 a_w values and subjected to 600 MPa. For S12-1 strain, the initial downregulation was also diminished throughout the storage at 4 °C, mainly for *sigB* gene.

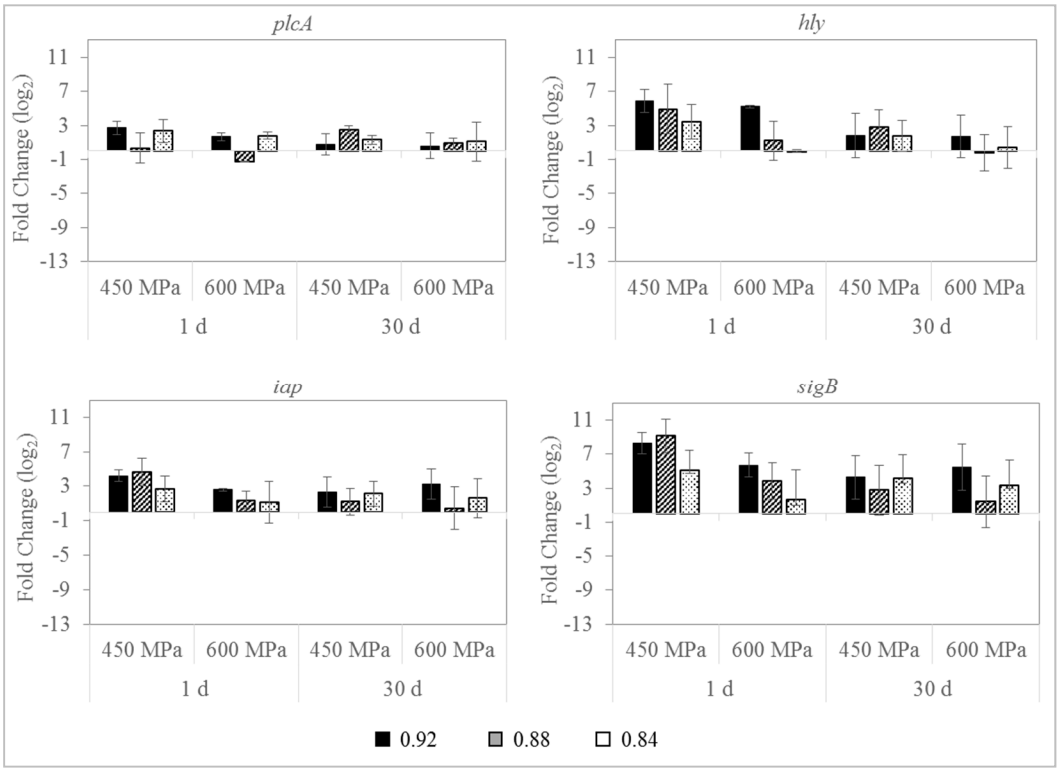

Figure 5. Relative changes in the transcription level for virulence (*plcA*, *hly*, and *iap*) and stress-related (*sigB*) genes of *L. monocytogenes* strain S4-2 (serotype 1/2b) in sliced dry-cured ham with different a_w values (0.92, 0.88, and 0.84), treated with HPP (450 MPa/10 min or 600 MPa/5 min) and stored during 30 days at 4 °C. Error bars indicate standard deviation of three biological replicates with duplicated samples.

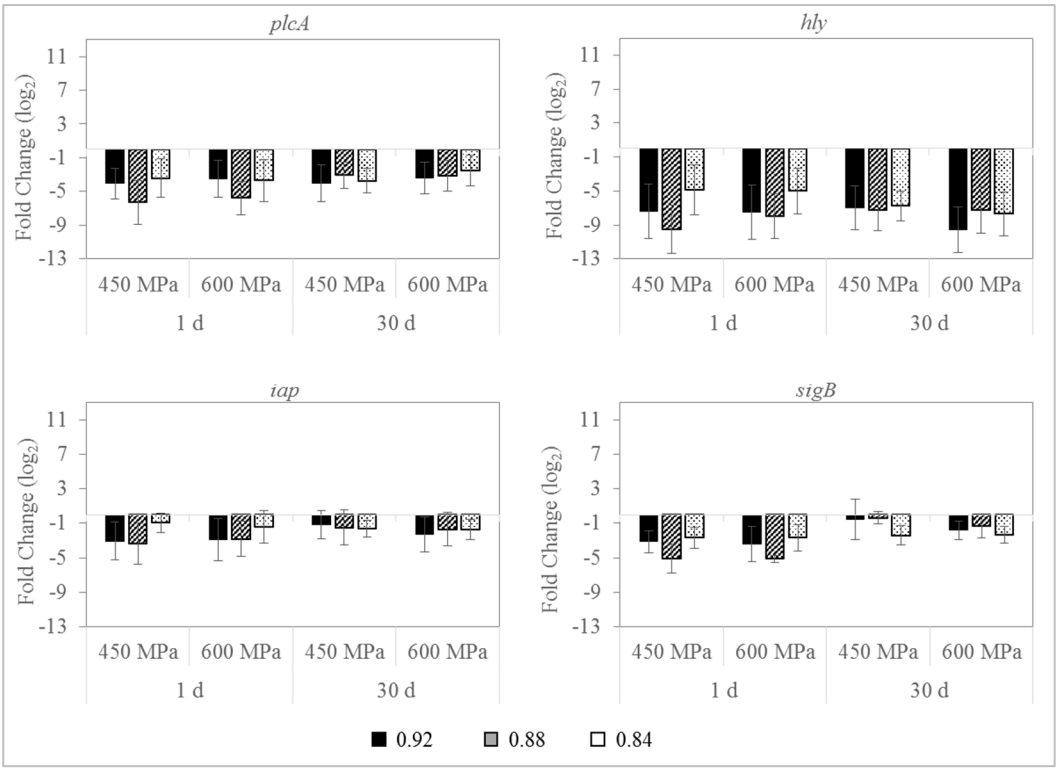

Figure 6. Relative changes in the transcription level for virulence (*plcA*, *hly*, and *iap*) and stress-related (*sigB*) genes of *L. monocytogenes* strain S12-1 (serotype 1/2c) in sliced dry-cured ham with different a_w values (0.92, 0.88, and 0.84) treated with HPP (450 MPa/10 min or 600 MPa/5 min) and stored during 30 days at 4 °C. Error bars indicate standard deviation of three biological replicates with duplicated samples.

4. Discussion

The water activity of dry-cured ham was modified to attain values of 0.92, 0.88, and 0.84, simulating hams of different aging times (drying, cellar, and finished products, respectively). Two strains of *L. monocytogenes* were subjected to HPP in dry-cured ham with different a_w values, strain S4-2 (serotype 1/2b) which was considered a persistent strain found in the environment and raw and final cured meat products and strain S12-1 (serotype 1/2c), which was non-persistent and isolated from dry-cured products [16]. Reductions on *L. monocytogenes* S4-2 population attained by 600 MPa/5 min were 2.5 log units in dry-cured ham with higher a_w values (0.92). Strain S12.1 resulted significantly ($p \leq 0.05$) more sensitive to HPP, with reductions higher than 2.8 log units. The efficacy of the treatments against *L. monocytogenes* diminished at lower a_w values mainly at 600 MPa/5 min. Thus, reductions in S4-2 and S12-1 population were equal or less than 1.0 and 1.6 log units, respectively, when 600 MPa/5 min treatments were applied to dry-cured ham with a_w values of 0.88 and 0.84. The efficacy of HPP to eliminate *L. monocytogenes* in dry-cured ham has been reported to be affected by physico-chemical properties of the food matrix such as a_w that could be very variable in this product. Thus, according to Bover-Cid et al. [23], dry-cured ham a_w can vary from 0.85 and 0.94. In vacuum-packed sliced dry-cured ham samples, up to 60% presented values of 0.92 or higher [24]. Low a_w values in dry-cured ham could protect microorganisms against HPP and diminish the bacterial inactivation caused by these treatments [25]. *L. monocytogenes* population was reduced 3.85 log units by 600 MPa/5 min in dry-cured hams with a_w values of 0.92, whereas reductions of 1.85 log units were obtained in hams with a longer drying and ripening period and a_w values of 0.88 [24]. This decrease in the antilisterial effect of HPP was also registered by Bover-Cid et al. [23], who observed reductions of 6.8 and 2.2 log units by 600 MPa/5 min in dry-cured ham with a_w values of 0.96 and 0.86, respectively, and by Morales

et al. [26], who reported higher inactivation of *L. monocytogenes* in Iberian dry-cured ham (a_w = 0.90) pressurized at 450 MPa/10 min than in Serrano ham (a_w = 0.88).

The predominant microorganisms in low a_w and high salt content foods as dry-cured ham are Gram-positive, catalase-positive cocci, as well as molds and yeasts [27]. Only microbial groups adapted to the low a_w values will survive during the ripening of dry-cured ham. In the present work, reductions on TVC ranged from 1.1 to 1.9 log units in dry-cured ham with a_w values of 0.92, 0.88, and 0.84 and subjected to 600 MPa/5 min, being greater at 0.92 a_w values. Moreover, bacterial counts increased in non-pressurized dry-cured ham with a_w values of 0.92 throughout the storage, whereas kept unchanged or even decreased in samples with a_w values of 0.88 and 0.84, respectively. Total aerobic counts were reduced by 1.2 log units in dry-cured ham with a_w values of 0.89 and treated at 600 MPa for 6 min [28]. Contrary, Martínez-Onandi et al. [29] concluded that a_w did not significantly change TVC in either untreated or dry-cured ham treated at 600 MPa/6 min.

Values of pH in dry-cured ham were barely affected as consequence of a_w modification or HPP, being the differences between samples lower than 0.2 pH units. Values of a_w neither affected nitrites nor NaCl content in dry-cured ham. Previous works have observed that HPP caused minor changes in the pH of dry-cured ham [30,31].

The influence of HPP on gene transcription patterns of *L. monocytogenes* in real food products has been scarcely studied. Moreover, the role of physicochemical properties of dry-cured ham such as a_w on the physiology of the pathogen has not been adequately approached. Therefore, one purpose of this work was to evaluate the impact of a_w on the relative expression of four genes involved in virulence and the stress response (*plcA*, *hly*, *iap*, and *sigB*) of *L. monocytogenes* inoculated in dry-cured ham and subjected to HPP. Moreover, relative gene transcription was compared for two strains of *L. monocytogenes* belonging to different serotypes. Our results suggest that changes in HPP-surviving bacteria gene transcription patterns were strain-dependent. In strain S4-2 (serotype 1/2b) the target genes *plcA*, *hly*, *iap*, and *sigB* were generally overexpressed by HPP in dry-cured ham at the three a_w values investigated, while in strain S12-1 (serotype 1/2c) the above genes were repressed. The differential behavior of *L. monocytogenes* strains was previously reported after the exposure of the pathogen to an enterocin extract or bacteriocin-producing *Enterococcus faecalis* in dry-cured ham [32], and when 3.5% of NaCl was used in a cheese-based medium to control *L. monocytogenes* [33].

L. monocytogenes potential to respond to adverse conditions or changes in the environment is mediated by various alternative sigma (σ) factors. The alternative sigma factor, SigB (σ^B), allows *L. monocytogenes* to multiply and survive under stress conditions in non-host associated environments [34], including those encountered in foods such as acidic or osmotic conditions [13,35]. In this work, an upregulation of *sigB* gene expression was observed for strain S4-2 in pressurized dry-cured ham with different a_w, being such induction smaller at lower a_w values. In this way, an increase of the *sigB* expression levels was reported in dry-cured fermented sausages, with decreasing values of pH and a_w during the manufacturing process [36]. Similarly, the activity of the SigB of *L. monocytogenes* was induced when the pathogen was exposed to 3% NaCl (≈ 0.96 − 0.97 a_w) [37]. Contrary, a downregulation of *sigB* was recorded for strain S12-1, being the expression also less pronounced in samples with the lowest a_w values. HPP-surviving cells could be highly injured, which may lead to increase in the expression of cell damage repair mechanisms and to repress virulence genes [38], mainly in dry-cured ham with very low a_w values.

SigB contributes to overcome food processing such as HPP [13], is fundamental in stress regulation and mediates the transcription of PrfA, the major regulator of *Listeria* virulence. The repression of *sigB* and *prfA* genes was observed for *L. monocytogenes* S12-1 when 450 MPa/10 min or 600 MPa/5 min were applied in dry-cured ham with different a_w values. Similarly, Bowman et al. [38] reported the suppression of these genes for a *L. monocytogenes* strain belonging to serotype 1/2a when HPP at 400 or 600 MPa for 5 min was applied on TSYE broth. On the contrary, *sigB* gene expression was increased after HPP for strain S4-2, but such initial overexpression was attenuated throughout the storage at 4 °C.

PrfA mediates the transcription of several virulence genes, including *plcA* and *hly*. Thus, a downregulation of *sigB* gene could result in a repression of *prfA* and, consequently, in reducing levels of *plcA* and *hly*. This fact could explain the trend of expression of these genes, which was similar to that observed for *sigB* gene. Immediately after pressure treatments, *hly* and *plcA* genes were upregulated for S4-2 strain in dry-cured ham with 0.92, 0.88, and 0.84 a_w values and repressed for S12-1. Same tendency was registered for *iap* gene, which encoded the invasion-associated protein p60 and is involved in the invasion of mammalian cells [39].

L. monocytogenes S4-2 and S12-1 responded differently to HPP. Strain S12-1 was slightly more sensitive to pressurization treatments than strain S4-2. HPP-surviving cells of strain S12-1 could result in more injured and this might have led to increased expression of cell damage repair genes but not virulence or stress-related genes. In the same way, pressure treatments at 400 or 600 MPa for 5 min caused a downregulation of genes associated with growth and virulence, such as *hly* and *iap*, for a *L. monocytogenes* strain (serotype 1/2a) in TSYE broth [38]. Differences in response to HPP and gene expression between strains might be related with serotype and/or other characteristics as persistence in the industrial environment. Further studies would be necessary to elucidate the differences observed in the present work.

5. Conclusions

L. monocytogenes strains S4-2 (serotype 1/2b) and S12-1 (serotype 1/2c) were reduced greatly by HPP in sliced dry-cured ham with a_w values of 0.92, mainly at 600 MPa/5 min. The efficacy of the HPP treatments diminished at lower a_w. Pressure treatments and a_w values of dry-cured ham changed the expression patterns of four virulence and stress-related genes (*plcA, hly, iap,* and *sigB*) but the behavior of two strains of the pathogen was different. *L. monocytogenes* S4-2 exhibited an upregulation of the target genes involved in virulence and stress-related, mainly in dry-cured ham with a_w values of 0.92, which was attenuated during refrigerated storage. Strain S12-1 showed a downregulation of all genes tested after HPP, mainly for *hly* and *plcA*. This study highlights that HPP effectiveness against *L. monocytogenes* could be reduced by low a_w values and that gene expression may be influenced by HPP and a_w values in dry-cured ham. However, no growth of HPP-survival cells was observed during refrigerated storage in low-a_w dry-cured ham, and the overexpression of virulence and stress-related genes diminished. The a_w values and other physicochemical properties such as NaCl or nitrites content in dry-cured ham is very variable and could hardly influence the stress adaptation mechanisms of the pathogen. Therefore, a better understanding of the role of such physicochemical properties of dry-cured ham on the physiology of *L. monocytogenes* should be further evaluated.

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Conflicts of Interest: The authors declare no conflict of interest.

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Effect of Enterocins A and B on the Viability and Virulence Gene Expression of *Listeria monocytogenes* in Sliced Dry-Cured Ham

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Abstract: Dry-cured ham can be contaminated with *Listeria monocytogenes* during its industrial processing. The use of bacteriocins could ensure the safety of such meat products, but their effect on pathogen physiology is unknown. Therefore, the impact of enterocins A and B on the *L. monocytogenes* population, and the expression patterns of five genes (*inlA*, *inlB*, *clpC*, *fbpA* and *prfA*) related to adhesion/invasion and virulence regulation have been monitored in sliced dry-cured ham during 30 d of storage in refrigeration (4 °C) and temperature-abuse conditions (20 °C). *L. monocytogenes* strains S2 (serotype 1/2a) and S7-2 (serotype 4b) counts were reduced by 0.5 and 0.6 log units immediately after the application of enterocins A and B, a decrease lower than previously reported. Differences in gene expression were found between the two strains. For strain S2, expression tended to increase for almost all genes up to day seven of storage, whereas this increase was observed immediately after application for strain S7-2; however, overall gene expression was repressed from day one onwards, mainly under temperature-abuse conditions. *L. monocytogenes* strains investigated in the present work exhibited a mild sensitivity to enterocins A and B in sliced dry-cured ham. Bacteriocins caused changes in the expression patterns of virulence genes associated with adhesion and invasion, although the potential virulence of surviving cells was not enhanced.

Keywords: pathogen; bacteriocin; cured meat; qPCR; invasion ability; virulence

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1. Introduction

Listeria monocytogenes is a food-borne pathogenic bacteria, which causes a serious disease called listeriosis with one of the highest hospitalization rates in developed countries (more than 90% of cases), affecting mainly susceptible groups such as new-born infants, children, pregnant women, elderly and immunocompromised individuals [1]. Listeriosis has been associated with a case-fatality rate of 17.6% in the European Union during 2019 [1]. Contaminated food is the major source of infection, and the gastrointestinal tract is the primary site of entry for the pathogen [2]. After adhesion to the host cell by different factors [3,4], two invasion proteins, internalins A and B (InlA and InlB), are fundamental in the internalization of the bacterium [5]. PrfA, considered the major virulence factor of *L. monocytogenes*, positively regulates the transcription of several virulence genes, including *inlA* and *inlB* [6].

Ready-to-eat (RTE) foods have been most frequently implicated in listeriosis outbreaks [1]. Meat, fish and dairy products are commonly associated with human infections, although foods of plant origin or frozen foods have also been involved [7]. Dry-cured ham is an RTE meat product considered safe due to its reduced water activity (a_w) and high salt content [8,9], but can be contaminated with *L. monocytogenes* during post-processing [10,11]. *L. monocytogenes* has been detected in dry-cured ham processing environments [12,13] and, despite cleaning and disinfection procedures, the pathogen could persist and reach the final product.

The microbiological criteria for *L. monocytogenes* in the EU established a maximum of 100 CFU/g for RTE foods, other than those intended for infants and medical purposes, and those that do not support the growth of the pathogen [14]. In contrast, the USA has a "zero tolerance" approach (absence in 25 g) for all RTE foods [15]. Additional control measures, such as high pressure processing or antimicrobial agents, could be necessary to ensure the safety of food products and avoid the economic losses due to the most restrictive regulatory requirements. Furthermore, the study of changes in gene expression upon exposure of *L. monocytogenes* to post-processing antimicrobial treatments in food could contribute to understand the response of the pathogen to different inactivation strategies. Biopreservatives, such as lactic acid bacteria (LAB) and/or their metabolites, have received considerable interest in the control of food-borne pathogens as an antimicrobial hurdle in foods and food-processing facilities. Bacteriocins produced by *Enterococcus* spp. exhibit antimicrobial activity against food-borne pathogens and have been explored in the control of *L. monocytogenes* in different meat products [16–18]. Enterocins modified the stress response or adaptation of *L. monocytogenes* in dry-cured ham, with differences between the responses of serotypes 1/2b and 1/2c [19]. Although the presence of enterocins determined the downregulation of genes involved in acid and osmotic stress, this effect was more pronounced on the serotype 1/2c strain [19]. Nevertheless, the knowledge of the effect of bacteriocins on *L. monocytogenes'* relative expression patterns of virulence genes related with adhesion and invasion in foods is scarce. Thus, the purpose of this work was to evaluate the effect of an extract of enterocins A and B produced by *E. faecium* INIA TAB7 on the viability and the relative expression of genes involved in the virulence of two strains of *L. monocytogenes* (serotypes 1/2a and 4b) in sliced dry-cured ham, stored under a strict refrigeration temperature (4 °C) and temperature-abuse conditions (20 °C) for 30 days.

2. Materials and Methods

2.1. Microorganisms and Culture Conditions

L. monocytogenes strains S2 and S7-2, obtained from the environment of an Iberian pig processing plant (Spain) and previously characterized by Ortiz et al. [20], were used as target organisms. Strains S2 and S7-2 were serotypes 1/2a and 4b, the most common serotypes from meat industry and clinical samples, respectively. The strains were held as stock cultures at −80 °C in Brain Heart Infusion broth (BHI, Biolife s.r.l., Milano, Italy) supplemented with 20% glycerol. *E. faecium* INIA TAB7 [21] was used for enterocins A and B production. The strain was preserved as stock culture at −80 °C in De Man, Rogosa and Sharpe broth (MRS, Biolife, Milano, Italy) supplemented with 20% glycerol. Before use in experiments, *L. monocytogenes* strains or *E. faecium* INIA TAB7 were sub-cultured twice in BHI broth at 37 °C for 18 h or in MRS broth with Tween® 80 (Biolife) at 30 °C for 18 h, respectively.

2.2. Enterocins Extract

E. faecium INIA TAB7 grown in MRS broth with Tween® 80 for 18 h at 30 °C was used to obtain the enterocins A and B extract as previously described [22]. The antimicrobial activity was determined against the two strains of the pathogen through the agar spot test [23] and was expressed as arbitrary units (AU) per mL.

2.3. Dry-Cured Ham Samples

One large piece (~7 kg) of deboned dry-cured ham was purchased from a commercial supplier in Spain and aseptically sliced in the laboratory. Slices of 5 g were inoculated by adding a cell suspension of *L. monocytogenes* S2 or S7-2 on the surface of the dry-cured ham to attain a final concentration of ca. 10^6 CFU/g. Cell suspensions were prepared from overnight cultures in BHI broth and their concentration was evaluated by plating on duplicate plates of CHROMagar *Listeria* (CH-L, Scharlab S.l., Barcelona, Spain). Enterocins A and B extract was added on the surface of sliced dry-cured ham to achieve a final activity of 1054 AU/g. Dry-cured ham samples were vacuum-packaged in BB325 bags

(200 mm × 300 mm, Cryovac Sealed Air Corporation, Milan, Italy) and stored at 4 or 20 °C for 30 d. Sliced dry-cured ham inoculated with either of the two *L. monocytogenes* strains but without enterocins was used as control. Three independent experiments were carried out.

2.4. L. monocytogenes Enumeration

L. monocytogenes counts were determined immediately after the enterocins A and B extract application and at 1, 7, 14 and 30 d of storage. Samples of dry-cured ham were diluted 10-fold with sterile 0.1% (*wt/vol*) peptone water solution and homogenized for 120 s using a Silver Masticator homogenizer (IUL Instruments, Barcelona, Spain). *L. monocytogenes* counts were determined on duplicate plates of CH-L, incubated at 37 °C for 48 h.

2.5. RNA Extraction and Retrotranscription

RNA extraction was carried out at 0 and 6 h and 1, 7 and 30 d after adding the enterocins A and B extract, according to the procedure described by Rantsiou et al. [24] with some modifications. Samples were diluted and homogenized as described in Section 2.4. Four milliliters of the homogenates were centrifuged at 10,000× *g* for 5 min and 50 µL of RNAlater (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) was added to the pellet. Samples were treated with 50 µL of lysozyme (50 mg/mL; Sigma-Aldrich) and incubated at 37 °C for 20 min in a Thermomixer compact (Eppendorf Scientific, Hamburg, Germany). Total RNA was extracted using the MasterPureTM complete DNA and RNA purification kit (Epicentre, Madison, WI, USA) following the instructions of the manufacturer. Residual DNA was digested using the Turbo DNase (Invitrogen, Thermo Fisher Scientific, Walthman, MA, USA) and complete removal of the DNA was verified by quantitative PCR (qPCR), as described in Section 2.6. Then, RNA quantity and quality were determined using a NanoPhotometer (Implen N60, Thermo Fisher Scientific) and normalized to 100 ng/µL. cDNA was obtained using the GoScriptTM Reverse Transcription Mix, Random Primers (Promega, Madison, WI, USA), according to the manufacturer's instructions, and was stored at −20 °C until use.

2.6. L. monocytogenes Relative Gene Expression

Five genes (*inlA*, *inlB*, *clpC*, *fbpA* and *prfA*), representative of *L. monocytogenes* virulence and previously used in studies of gene expression [24–28], were amplified by qPCR (Table 1). Further, IGS was selected as a reference gene and internal control. Three biological replicates were analyzed in a 96-well plate (VWR International, Radnor, PA, USA) for each gene of interest and each sample was amplified in duplicate. *L. monocytogenes* DNA control sample, together with a template-free negative control, were also included in the runs. Plates were sealed with optical adhesive covers (Bio-Rad Laboratories, Hercules, CA, USA). In order to minimize the variance introduced by the instrument between the runs (inter-runs), all the samples belonging to the same strain and temperature were assayed for each gene separately in the same plate. The qPCR assays were carried out using the Mx3000P Real-Time PCR system (Agilent Technologies, Santa Clara, CA, USA), with the use of GoTaq® Probe qPCR Master Mix (Promega, Madison, WI, USA). Reactions (final volume of 25 µL) contained: 12.5 µL of the 2X GoTaq® Probe qPCR Master Mix, 0.9 µM (*inlA*, *inlB*, *clpC*, *fbpA* and *prfA*) or 0.4 µM (*IGS*) of each primer, 0.25 µM (*inlA*, *prfA* and *IGS*) or 0.20 µM (*inlB*, *clpC* and *fbpA*) of the probe and 2 µL of cDNA template. The amplification program consisted of one cycle at 95 °C for 3 min, followed by 40 cycles of 15 s at 95 °C and 30 s at 50 °C (*fbpA*), 30 s at 60 °C (*inlB*, *clpC*) or 1 min at 60 °C (*inlA*, *prfA* and *IGS*). The PCR efficiency of each primer pair was previously determined using 10-fold dilutions of genomic DNA extracted from both *L. monocytogenes* strains as a template and adequate amplification efficiencies for target and reference genes were obtained. Threshold cycle (C_T) values from qPCR were used for relative quantification.

Table 1. *L. monocytogenes* genes targeted by qPCR in this study to determine the effect of enterocins A and B on adhesion/invasion and virulence gene expression.

Gene Name	Function and Scope of Use	Sequence (5′→3′)	Reference
IGS	Reference gene	IGS1: GGCCTATAGCTCAGCTGGTTA IGS2: GCTGAGCTAAGGCCCCATAAA P: HEX-CCATCGACCTCACGCTTATCAGGC-TAMRA	[24] [25]
inlA	Internalization in the host cell	F: GGTCTCACAAACAGATCTAGACCAAGT R: TCAAGTATTCCACTCCATCGATAGATT P: HEX-TCCCTAATCTATCCGCCTGAAGCGTTG-TAMRA	[26]
inlB	Internalization in the host cell	F: AAGCAAGATTTCATGGGAGAGT R: TTACCGTTCCATCAACATCATAACTT P: HEX-CCACTGAAAGAGGTTTACACA-TAMRA	[27]
clpC	ATPase involved in cell adhesion and invasion	F: GCGGCTGTTCAAGGTCAAG R: TTGCCAATTCGCTTTAGTTTCTT P: HEX-AAAGCAGCGTCATTACG-TAMRA	[27]
fbpA	Involved in efficient colonization of host tissues	F: AAATCAATGAACTATTTCCGGAAAG R: CATGGAGCTTGCTAAAC P: HEX-CTAGAGGAGCATAAGGAA-TAMRA	[27]
prfA	Transcriptional regulator, virulence	F: CAATGGGATCCACAAGAATATTGTAT R: AATAAAGCCAGACATTATAACGAAAGC P: HEX-TGTAAATTCATGATGGTCCCGTTCTCGCT-TAMRA	[28]

F, forward; R, reverse; P, probe; HEX, fluorochrome at 5′-end of the probe; TAMRA, quencher of HEX at 3′-end of the probe.

2.7. Data and Statistical Analysis

Relative gene transcription levels (fold changes) were calculated by the $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta C_T$ is: $(C_{T\text{target}} - C_{T\text{reference gene}})_{\text{test condition}} - (C_{T\text{target}} - C_{T\text{reference gene}})_{\text{control condition}}$ [29]. Virulence genes were considered targets, while *IGS* was considered a reference gene, the expression of which was considered constant regardless of the application of treatments. The test condition was the dry-cured ham inoculated with *L. monocytogenes* and treated with enterocins A and B, while control condition was the dry-cured ham without enterocins, at five different time points after treatments. Log_2 values of relative expression were obtained.

Statistical treatment of log_2 values of relative gene expression was carried out by means of SPSS Statistics 22.0 software (IBM Corp., Armonk, NY, USA). The significant differences between *L. monocytogenes* counts were also evaluated. The Tukey test was applied to detect significant differences between means at $\alpha = 0.05$.

3. Results and Discussion

3.1. Effect of Enterocins on L. monocytogenes Population

The antimicrobial activity of enterocins A and B, determined against the two strains of the pathogen through the agar spot test, was estimated to be 51,200 AU/mL. *L. monocytogenes* counts in control and enterocins A- and B-treated sliced dry-cured ham stored at 4 and 20 °C during 30 d are shown in Table 2. Initial counts in the control ham ranged between 6.2 and 6.3 log CFU/g for *L. monocytogenes* S2 and S7-2. Immediately after the application of enterocins A and B, S2 and S7-2 counts were significantly ($p < 0.05$) reduced by 0.5 and 0.6 log units, respectively, at both temperatures. During the storage, *L. monocytogenes* S2 counts in enterocin treated samples decreased by 1.6 and 1.9 log units at 4 and 20 °C, respectively, whereas S7-2 counts were reduced by 1.9 and 1.2 log units. At the end of the storage period at 4 and 20 °C, *L. monocytogenes* S2 counts were significantly lower in dry-cured ham treated with enterocins A and B than in control samples, whereas this difference was significant ($p < 0.05$) only for *L. monocytogenes* S7-2 at 4 °C.

Table 2. *L. monocytogenes* S2 and S7-2 counts (log CFU/g) in sliced dry-cured ham treated with enterocins A and B and stored during 30 d at 4 and 20 °C.

Strain	Temperature (°C)	Treatment	Time (d)				
			0	1	7	14	30
S2	4	Control	6.29 ± 0.18aD	5.61 ± 0.16aC	5.18 ± 0.14aB	5.26 ± 0.20aB	4.84 ± 0.32aA
		ENT	5.80 ± 0.15bE	5.20 ± 0.17bD	4.90 ± 0.09bC	4.59 ± 0.15bB	4.16 ± 0.13bA
	20	Control	6.18 ± 0.09aD	5.63 ± 0.20aC	5.07 ± 0.09aB	4.80 ± 0.27aB	4.21 ± 0.25aA
		ENT	5.67 ± 0.27bC	4.84 ± 0.12bB	4.63 ± 0.29bB	4.08 ± 0.42bA	3.81 ± 0.29bA
S7-2	4	Control	6.25 ± 0.11aD	5.84 ± 0.08aC	5.46 ± 0.17aAB	5.51 ± 0.13aB	5.26 ± 0.13aA
		ENT	5.63 ± 0.18bB	5.26 ± 0.09bB	4.84 ± 0.37bA	4.86 ± 0.33bA	4.69 ± 0.13bA
	20	Control	6.25 ± 0.20aC	5.72 ± 0.28aBC	5.38 ± 0.33aB	5.18 ± 0.33aAB	4.72 ± 0.53aA
		ENT	5.67 ± 0.12bB	5.27 ± 0.10bB	4.35 ± 0.32bA	4.27 ± 0.83bA	4.49 ± 0.18aA

Control, non-treated. ENT: treated with an enterocins A and B extract produced by *E. faecium* INIA TAB7. Values are the mean ± SD. a, b Means within the same column with different lowercase letters differ significantly at $p < 0.05$ for a given strain and temperature. A, B, C, D, E Means within the same row with different uppercase letters differ significantly at $p < 0.05$.

The potential of bacteriocins to control *L. monocytogenes* has been previously investigated in dry-cured ham. Nisin exhibited a bactericidal effect against *L. monocytogenes* immediately after its application on the surface of dry-cured ham slices and such antilisterial activity was maintained during 2 months of storage at 8 °C, being more pronounced in dry-cured ham with lower a_w [30]. Sakacin K and enterocins A and B also induced significant reductions in the level of the pathogen in dry-cured ham 1 d after application [31]. This antilisterial effect was also observed for enterocins A and B in dry-cured ham against a four-strain cocktail of *L. monocytogenes*, with reductions higher than 2 log units during 30 d of storage at 4 °C [18]. Our results confirm the activity of enterocins A and B against *L. monocytogenes*. However, the bactericidal efficacy was lower in the present work, a fact that could be attributed to differences in the sensitivity of the enterocins among different *L. monocytogenes* strains. Similar results were reported by Montiel et al. [19], with differences between two *L. monocytogenes* strains belonging to different serotypes (1/2b and 1/2c). Different behavior between different serotypes was also recorded after the application of other antilisterial bacteriocins [32,33].

3.2. Effect of Enterocins on L. monocytogenes Gene Expression

The relative gene transcription profiles of five representative virulence genes (*inlA*, *inlB*, *clpC*, *fbpA* and *prfA*) of *L. monocytogenes* S2 and S7-2 strains, induced by enterocins A and B during 30 d of storage at 4 or 20 °C, are shown in Figures 1 and 2, respectively. Different gene expression profiles between the two strains were detected. Specifically, a slight upregulation for *inlA* and *inlB* was observed for strain S2 immediately after the application of the enterocins at both temperatures, whereas a downregulation was recorded for *prfA* and *clpC* genes, although differences between control and treated samples were not statistically significant. For strain S7-2, an overall upregulation for almost all target genes was observed immediately after enterocins extract application. Our results point out that changes in the surviving bacteria gene transcription profiles were different between the two strains. This fact was observed after the exposure of the pathogen to enterocins or bacteriocin-producing *E. faecalis* B1 in dry-cured ham [19]. Differences in gene expression between *L. monocytogenes* strains have also been reported after high pressure processing [25,34], mild heat shock stress [35], or in the presence of different levels of salt in a simulated cheese medium [36], a dry-cured ham model system [37] or liver pâtés [27]. Further studies would be necessary to elucidate if differences in the cellular response induced by antimicrobial treatments or food conditions could be associated with serotype or with other strain characteristics.

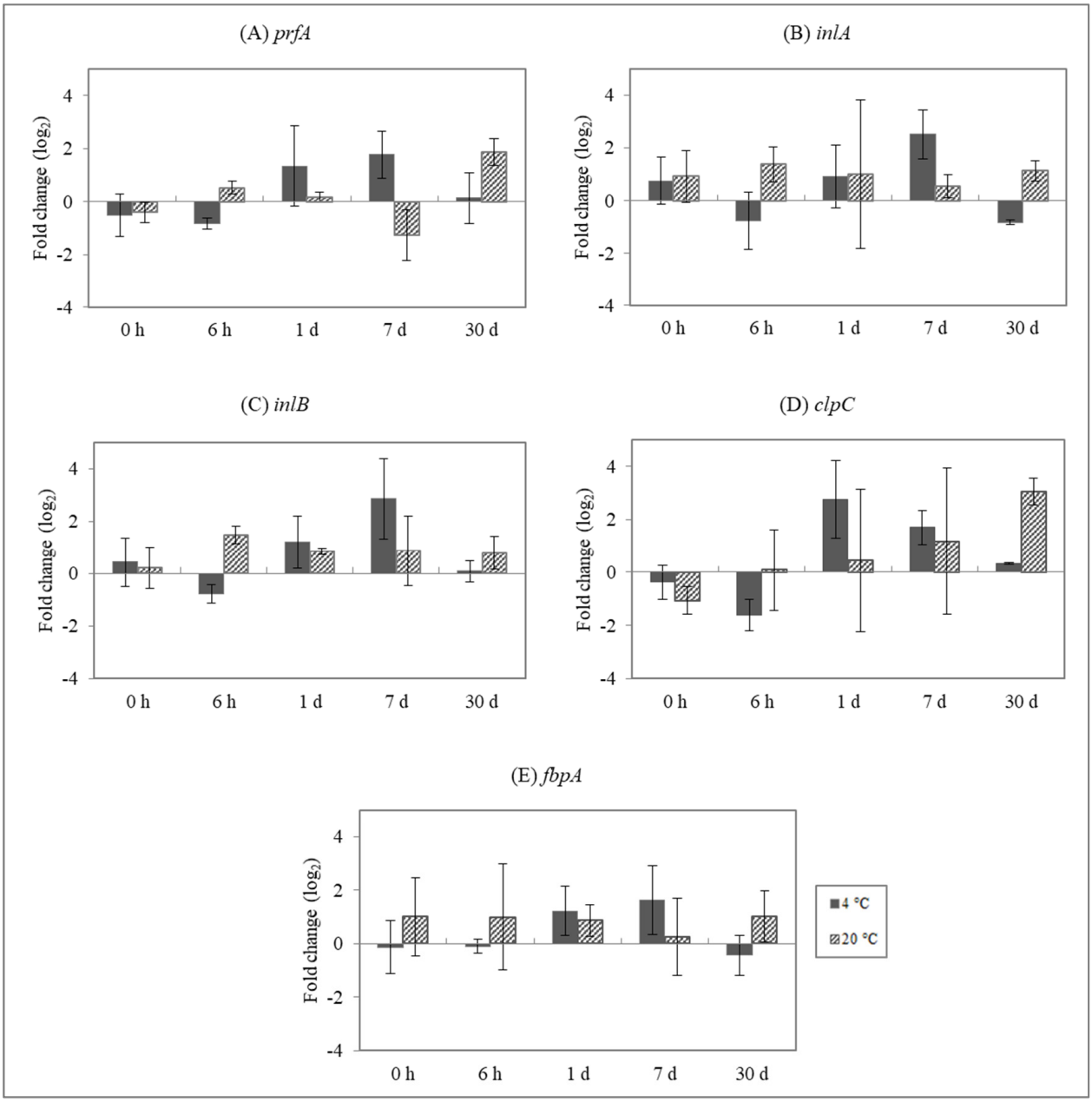

Figure 1. Relative change in the transcription level for five virulence genes *prfA* (**A**), *inlA* (**B**), *inlB* (**C**), *clpC* (**D**) and *fbpA* (**E**) of *L. monocytogenes* strain S2 in sliced dry-cured ham treated with enterocins A and B and stored during 30 d at 4 and 20 °C. Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method and $\log_2$ values are reported. Error bars indicate standard deviation of three biological replicates with duplicated samples (n = 6).

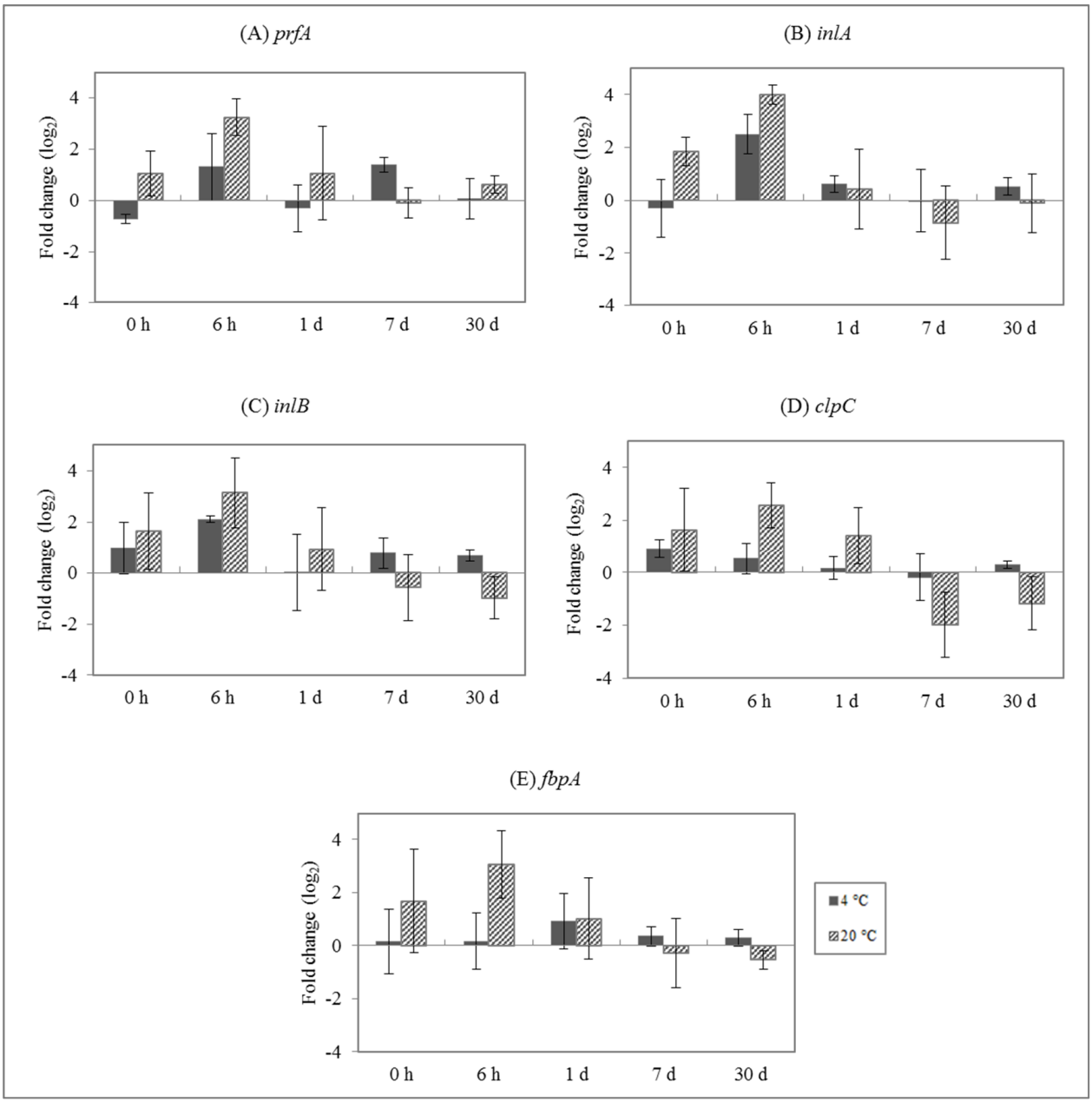

Figure 2. Relative change in the transcription level for five virulence genes *prfA* (**A**), *inlA* (**B**), *inlB* (**C**), *clpC* (**D**) and *fbpA* (**E**) of *L. monocytogenes* strain S7-2 in sliced dry-cured ham treated with enterocins A and B and stored during 30 d at 4 and 20 °C. Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method and $\log_2$ values are reported. Error bars indicate standard deviation of three biological replicates with duplicated samples (n = 6).

The expression of the target genes fluctuated during refrigerated storage and such changes were affected by temperature. For strain S2, an overall upregulation trend was recorded throughout the storage in treated dry-cured ham. At 4 °C, the upregulation was registered up to day 7, followed by a decrease in expression. At 20 °C, all target genes increased their expression during the 30 d of storage, this increase being statistically significant ($p < 0.05$) for *clpC* and *fbpA* genes, both related to the adhesion and invasion of *L. monocytogenes*. For strain S7-2, the initial overexpression recorded for all genes was maintained only during the first 6 h of storage and was reduced afterwards, being more pronounced in samples stored at 20 °C after 30 d. Changes to *L. monocytogenes* gene expression profiles caused as a function of the storage time were also observed

in dry-cured ham when an E-beam treatment at 3 kGy was applied [38], or when the pathogen was exposed to enterocins or co-cultured with a bacteriocin-producing *E. faecalis* for 7 d at 7 °C [19]. Specifically, these authors observed that the expression patterns of strains *L. monocytogenes* S4-2 and S12-1 fluctuated during the 7 d of storage at 7 °C. Regarding temperature, Rantsiou et al. [24] reported differences in the expression patterns of virulence and stress resistance genes of *L. monocytogenes* in different foods. Duodu et al. [39] concluded that exposure to temperature abuse conditions could affect potential virulence of low pathogenic *L. monocytogenes* strains in salmon. In this work, strain S2 tended to increase the expression of the target genes at the end of storage at 20 °C, although the changes recorded were not significant.

The effect of inactivation treatments on the virulence of *L. monocytogenes* in real food matrices has been barely investigated. Thus, the expression patterns of virulence and stress related genes of *L. monocytogenes* in dry-cured ham were increased by E-beam treatments [38], whereas they were slightly changed by high pressure treatments [25]. Regarding bacteriocins or bacteriocin-producing microorganisms, the expression patterns of some stress-related genes of *L. monocytogenes* in co-culture with a nisin producing *Lactococcus lactis* subsp. *lactis* in reconstituted skim milk at 20 and 30 °C for 24 h were modified [40]. Ye et al. [41] observed that a bacteriocinogenic *E. faecium* strain decreased the expression of most of the *L. monocytogenes* target genes assayed in a liquid culture medium at 4 °C, and Montiel et al. [19] reported the downregulation of some representative genes of stress response (*lmo2434*, *lmo0669*, *lmo1421* and *gbuB*) and the virulence regulatory gene *prfA* by addition of enterocins or enterocinogenic *E. faecalis* B1 in dry-cured ham inoculated with *L. monocytogenes* S4-2 and S12-1. On the contrary, in this work, an initial upregulation of *inlA* and *inlB* for strain S2 and of almost all target genes (*inlA*, *inlB*, *clpC* and *fbpA*) for strain S7-2 was observed after the addition of enterocins A and B, followed by a repression. The strains tested by Montiel et al. [19] resulted in being more sensitive to enterocins, suffering greater sublethal damage and, consequently, increasing the expression of cell damage repair genes and reducing the stress response and virulence genes expression, as previously indicated by Bowman et al. [42]. Furthermore, the possible development of resistance by *L. monocytogenes* in the presence of sublethal concentrations of enterocins should be considered. Laursen et al. [43] concluded that several *L. monocytogenes* genes known or speculated to be involved in the development of bacteriocin resistance showed increased expression when the pathogen was exposed to a pediocin-containing *Lactobacillus plantarum* supernatant.

The transcriptional factor PrfA is the major regulator of the pathogen virulence and mediates the transcription of several virulence genes, including *inlA* and *inlB*, which encode the two main proteins involved in host cell entry, particularly in non-phagocytic cells [5,6]. The PrfA-dependent expression is regulated by PrfA concentration as well as its affinity for the promoter. In this work, a repression of the *prfA* gene was observed for strains S2 and S7-2 immediately after treatment. In accordance with our results, an initial downregulation tendency was also observed after the addition of enterocins in dry-cured ham inoculated with *L. monocytogenes* S4-2 and S12-1 [19]. A downregulation of the *prfA* gene could result in a lower concentration of the PrfA factor and, consequently, in a minor transcription of *inlA* and *inlB* genes. However, an overall upregulation for such genes was detected. The presence of additional PrfA-independent promoters for *inlA* and *inlB* genes may contribute to the differential expression of PrfA-dependent genes, despite being controlled by PrfA [6]. At the end of storage period, the expression levels of the *prfA* and *inlA* and *inlB* genes followed a similar trend for strain S2, while for strain S7-2, the expression of the genes coding for the two internalins was more similar to *clpC*. The ClpC ATPase, encoded by *clpC*, also regulates the expression of the internalins A and B and is required for adhesion and invasion processes [4]. The expression pattern recorded for this gene is opposite for the two strains tested. Additionally, a different pattern depending on the strain was also recorded for the *fbpA* gene, coding for another adhesion-related molecule, especially regarding hepatocytes [44].

This paper provides additional information on *L. monocytogenes* virulence and invasiveness potential in a real food matrix. It is worth mentioning that serotype 1/2a is overrepresented among isolates from food environments, whereas serotype 4b predominates among isolates from human listeriosis cases. Furthermore, many *L. monocytogenes* serotype 1/2a strains widely characterized from the processing plants' environments present premature stop codons (PMSCs) in their *inlA* gene sequence [45], associated with virulence attenuation. In fact, strain S2 used in this work possessed PMSC6, while S7-2 showed a complete internalin sequence [46]. The information obtained in this study might be complemented by data from adhesion and invasion capacity using human intestinal cell lines. This would confirm whether the results obtained at the transcriptome level correlate with cell culture results, and the invasion capacity of the surviving cells would not be affected by the treatments.

4. Conclusions

L. monocytogenes strains S2 and S7-2 artificially inoculated in dry-cured ham exhibited a mild sensitivity to enterocins A and B during 30 d of refrigeration or under temperature-abuse conditions. The addition of enterocins affected the expression pattern of five adhesion/invasion and virulence genes (*inlA*, *inlB*, *clpC*, *fbpA* and *prfA*) with differences among the two strains investigated. S2 (serotype 1/2a) exhibited an overall upregulation trend up to day 7 of storage. Gene expression of strain S7-2 (serotype 4b) was initially induced by enterocins A and B, and was repressed from day 1 onwards. This study highlights that gene expression may be influenced by bacteriocins, although the virulence of surviving *L. monocytogenes* cells was not potentially enhanced by this antimicrobial. Based on all this, it can be concluded that enterocins A and B might be considered an interesting biological strategy to control *L. monocytogenes* in case of contamination during the post-processing of dry-cured ham even under temperature-abuse conditions. Further studies should combine gene expression with adhesion and invasion capacity of treated *L. monocytogenes* on intestinal cell lines.

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Discusión general

La presente tesis doctoral se desarrolla en una línea de investigación centrada en conocer el impacto de *Listeria monocytogenes* en jamón curado. La incidencia creciente de listeriosis, junto con el hecho de que la mayor tasa de mortalidad asociada al consumo de alimentos se debe a la combinación de *L. monocytogene*s y productos cárnicos, pone de manifiesto la necesidad de encontrar soluciones frente a este patógeno en el sector cárnico español, el cuarto más importante del país en términos económicos. Las características intrínsecas del jamón curado como su baja actividad de agua (a_w), generalmente en torno a 0,90, y alto contenido de sal, hacen que sea un producto RTE estable y con una larga vida útil a temperaturas de refrigeración, por lo que no está generalmente asociado con problemas de seguridad alimentaria (Reynolds et al., 2001; Serra-Castelló et al., 2020). No obstante, se han notificado algunas alertas por *L. monocytogenes* en jamón curado, como la comunicada por el Sistema de Alerta Rápida para Alimentos y Piensos de la Comisión Europea (FSANZ, 2016), con presencia de hasta 340 UFC/g del patógeno en jamones curados de origen italiano, francés y español. También se han retirado del mercado jamones alemanes curados y ahumados por posible contaminación con *L. monocytogenes* (AESAN, 2019). Por todo ello, estudiar la presencia del patógeno en las plantas de producción de jamón curado, así como caracterizar alguna de sus propiedades, permitirá no solo determinar el posible riesgo asociado a la presencia de la bacteria, sino también a su virulencia potencial, y desarrollar estrategias de control en caso de que fuera necesario. No obstante, la erradicación de *L. monocytogenes* de las plantas de procesado es prácticamente imposible, por lo que es preciso contar con tratamientos no térmicos de inactivación para controlar la presencia de este patógeno en caso de que alcance el producto final, al tiempo que se mantengan sus propiedades inalteradas. Dado que estos tratamientos de inactivación, aunque efectivos, pueden resultar incapaces de eliminar toda la carga microbiana, se ha propuesto en el presente trabajo profundizar en el comportamiento de las células supervivientes y/o dañadas subletalmente mediante el análisis transcriptómico de genes de resistencia a estrés y virulencia, considerado una herramienta eficaz para evaluar su impacto en un posible aumento de la patogenicidad de estas células en el jamón curado.

1. Caracterización de la contaminación por *L. monocytogenes* en industrias productoras de jamón curado

Aunque se ha demostrado la eliminación de *L. monocytogenes* en la superficie de jamones durante el proceso de curado (Montiel et al., 2020), la presencia del patógeno en

las plantas de producción de jamón puede dar lugar a contaminaciones en las etapas postproducción, como el deshuesado y/o loncheado. En este sentido, en un estudio previo realizado en 10 industrias españolas de elaboración de jamón curado se detectó la presencia de *L. monocytogenes* en un 2,7% de las muestras de producto final (D'Arrigo et al., 2020). En el trabajo desarrollado en esta tesis doctoral (Publicación 1), se ha investigado, además de la incidencia, algunas características fenotípicas y moleculares de las cepas que se encuentran en las zonas de deshuesado y loncheado de distintas industrias productoras de jamón curado, con el fin de obtener un mayor conocimiento del patógeno que servirá para mejorar los protocolos de limpieza y desinfección, así como para atajar una contaminación en caso de que se produzca, o realizar una mejor evaluación del riesgo potencial asociado a dicha contaminación.

Como se ha comentado anteriormente, *L. monocytogenes* se encuentra ampliamente distribuida, por lo que su entrada en las plantas de procesado de alimentos puede suceder a través de diversas fuentes como animales, materias primas, agua, materiales u operarios. Una vez dentro de la industria, esta bacteria es capaz de sobrevivir e incluso crecer con valores de pH tan bajos como 4,4, concentraciones de sal de hasta el 14% y en un amplio intervalo de temperaturas entre 1 y 45 ºC, algunas cepas incluso a temperaturas más bajas (Kramarenko et al., 2013), lo que hace posible el aislamiento de *L. monocytogenes* en dichos ambientes, como se discutirá posteriormente.

Existen diversos estudios en los que se ha investigado la incidencia de *L. monocytogenes* en productos cárnicos y las plantas en las que se producen y/o procesan (Chasseignaux et a., 2001; Martín et al., 2014; Møretrø et al., 2017; Sereno et al., 2019), con valores altos tanto en productos como en muestras ambientales. Más del 90% de los casos de contaminación de alimentos se deben a la contaminación cruzada entre estos y las superficies de contacto (Mazaheri et al., 2021).

En la presente tesis doctoral, se analizó la presencia de *L. monocytogenes* en las zonas de deshuesado y loncheado de tres plantas de procesado de jamón curado localizadas en tres zonas geográficas españolas diferentes. Se tomaron un total de 491 muestras de superficies de contacto y de no contacto de equipos e instalaciones, tanto durante la producción como tras los procesos de limpieza y desinfección, resultando positivas para *L. monocytogenes* el 11,8% de las muestras, todas recogidas de las zonas de deshuesado. Alía et al. (2020) detectaron *L. monocytogenes* en cinco de las seis plantas productoras de jamón curado que analizaron, con un 9,2% de muestras positivas, de las cuales el 6,7 y 2,1% se obtuvieron de las zonas de deshuesado y loncheado,

respectivamente. En las zonas de deshuesado de 43 plantas de producción de jamón curado de Parma se registró una prevalencia similar, del 10,1% (Morganti et al., 2016), siendo la incidencia mayor que en otras zonas postprocesado. Las operaciones mecánicas altamente invasivas que se realizan para extraer el hueso del jamón podrían explicar por qué las zonas de deshuesado son las contaminadas con mayor frecuencia con *L. monocytogenes*. La ausencia de *L. monocytogenes* en las zonas de loncheado analizadas en este estudio reducen potencialmente la probabilidad de contaminación de producto final loncheado. En el estudio de prevalencia de este patógeno en plantas españolas de jamón curado Serrano o Ibérico realizado por D'Arrigo et al. (2020), se detectó *L. monocytogenes* en el 19,4% de las muestras ambientales, un valor más alto debido probablemente a la toma de muestras a lo largo de la cadena de producción y no solo en zonas postprocesado.

En este trabajo, la prevalencia más alta del patógeno se registró en la planta B, con el 29,3% de muestras positivas, obteniéndose el 12,5% durante la producción y el 11,4% antes, tras la limpieza y desinfección. El 9,9% de las muestras analizadas resultó positivo para *L. monocytogenes* en la planta C, aislándose el patógeno durante y antes de la producción en el 7,5 y 2,4% de las muestras, respectivamente. En la planta A se registró el 8,0% de muestras positivas, el 5,3% se aisló durante la producción y el 2,7% tras los procesos de desinfección. Más de la mitad de las muestras positivas (51,7%) procedieron de superficies de no contacto con jamón, y, en general, solamente el 4,1% de las muestras positivas procedió de superficies tras la limpieza y desinfección, siendo dicho valor medio similar al obtenido por Alía et al. (2020) en zonas postprocesado de plantas de jamón curado y por debajo de los registrados en otras plantas de procesado de carne de cerdo, que aparecen en torno al 10% (D'Arrigo et al., 2020; Ortiz et al., 2010). Las diferencias registradas entre plantas podrían deberse al uso de distintos protocolos de limpieza por parte de las industrias implicadas en el estudio. El uso de mangueras para lavados con agua a alta presión puede hacer que *L. monocytogenes* se propague en líquidos o aerosoles por diversas zonas de la planta. El patógeno puede establecer nichos en zonas de difícil acceso para la desinfección, pudiendo estar sometido a concentraciones subletales de desinfectante que permiten el desarrollo de resistencias. En las industrias alimentarias, protocolos deficientes de limpieza, junto con algunos mecanismos que pueden ser desarrollados por los microorganismos, dificultan su eliminación. Por ello, más adelante se analizará la capacidad de formación de biofilms y la presencia de genes de resistencia a desinfectantes de algunas de las cepas aisladas de las industrias muestreadas, con el fin

de comprobar si presentan características feno- o genotípicas que contribuyan a su establecimiento y persistencia.

La mayoría de los aislados de *L. monocytogenes* detectados en las tres plantas fueron de serotipo 1/2a, con una incidencia general del 74,1%. Este serotipo también se registró con mayor frecuencia en otras industrias cárnicas (Chasseignaux et al., 2001; López et al., 2008; Martín et al., 2014; Ortiz et al., 2010). Los serotipos 1/2b y 1/2c correspondieron al 12,1% de los aislados, mientras que en la planta B se detectó el único aislado 4b del presente trabajo. Estos resultados coinciden con lo recogido en la literatura, donde se describe que las cepas del linaje II (serotipos 1/2a y 1/2c) se relacionan más a menudo con alimentos y plantas de procesado de alimentos, mientras que las cepas del linaje I (serotipos 1/2b y 4b) se relacionan normalmente con casos de listeriosis, aislándose con mayor frecuencia de muestras clínicas (Orsi et al., 2011). Mediante PFGE, los 58 aislados de *L. monocytogenes* se clasificaron en 20 pulsotipos o cepas diferentes. Algunas de estas cepas resultaron ser predominantes en cada una de las plantas y se aislaron tanto durante la producción como tras la limpieza y desinfección y de zonas de contacto y de no contacto, como por ejemplo A2 (58,3%), B4 (47,6%), C1 (20,0%) o C3 y C5 (16,0%), aunque se obtuvieron también pulsotipos que se consideraron esporádicos. No se detectó una misma cepa en plantas diferentes, aunque se identificaron pulsotipos similares genéticamente (en algunos casos con una similitud mayor al 95%) entre plantas separadas geográficamente, lo que indicaría la adaptación de ciertas cepas al ambiente de procesado de productos cárnicos. En este sentido, D'Arrigo et al. (2020) detectaron una misma cepa en tres plantas diferentes y otra en cuatro plantas de jamón curado. Cuatro de los pulsotipos (B4, C1, C3 y C5) obtenidos en este trabajo se encontraron repetidamente (tres veces o más) durante un período de al menos tres meses por lo que fueron considerados persistentes (Keto-Timonen et al., 2007), resultados que concuerdan con otros estudios en los que se habla de un número limitado de cepas persistentes pero una gran diversidad de cepas esporádicas (Alía et al., 2020; Bolocan et al., 2016; D'Arrigo et al., 2020; Ortiz et al., 2010). Se ha demostrado que las cepas de *L. monocytogenes* que contaminan los productos finales coinciden con las detectadas en los equipos de las plantas de procesado, lo que sugiere una contaminación ambiental por cepas que persisten en las instalaciones y el ambiente industrial (Colagiorgi et al., 2017; Keto-Timonen et al., 2007; Martínez-Suárez et al., 2016; McCollum et al., 2013) más frecuente que la procedente de las materias primas. Hay quien considera la persistencia como un fenómeno

esporádico, aunque también se ha asociado con una serie de factores, como la capacidad de formación de biofilms o la tolerancia a desinfectantes.

La nomenclatura a nivel de pulsotipo sigue normalmente un criterio establecido por el equipo que realiza el estudio, por lo que el uso de tipos de secuencia (ST) o complejos clonales (CC) resulta más útil a la hora de realizar comparaciones entre distintos trabajos. Los aislados obtenidos en este estudio se clasificaron en 11 tipos de secuencia y 10 complejos clonales, entre los que el ST121 y el ST9 fueron los más abundantes, lo que concuerda con su reconocida adaptación a industrias cárnicas (Martín et al., 2014). Concretamente, el ST121 se conoce por estar ampliamente distribuido en alimentos y plantas de producción de alimentos en toda Europa (Bergholz et al., 2018; Hein et al., 2011; Holch et al., 2013; Palma et al., 2017; Stoller et al., 2019). Esto puede deberse a que cepas con este ST suelen presentar la isleta de supervivencia a estrés 2 (SSI-2), que les permite adaptarse a condiciones de estrés alcalino y oxidativo ocasionadas por desinfectantes ampliamente utilizados en la industria alimentaria (Harter et al., 2017). Este ST, junto con el ST9, se consideran hipovirulentos y no se relacionan apenas con bacteriemia e infecciones del sistema nervioso central y materno-neonatal, todo lo contrario que el ST1 perteneciente al CC1 (Maury et al., 2016), tipo de secuencia al que pertenece la única cepa 4b aislada en este trabajo. Las cepas pertenecientes al CC1 se consideran hipervirulentas, ya que albergan las LIPI-1 y LIPI-4, relacionadas con la resistencia a arsénico, lo que contribuye a la virulencia, e incrementar la competitividad en el tracto gastrointestinal, respectivamente (Maury et al., 2016). Muchos de los aislados obtenidos en una de las plantas analizadas este trabajo (B) se adscribieron al ST87, el tercer tipo de secuencia más abundante. El ST87 se detectó también en productos cárnicos y en superficies de contacto de diferentes industrias cárnicas españolas (Martín et al., 2014), y se ha relacionado con dos brotes de listeriosis independientes en el norte de España (Pérez-Trallero et al., 2014). Wang et al. (2018) determinaron que el ST87 fue el tercer tipo de secuencia más abundante en muestras alimentarias de distintas provincias chinas, y también se ha encontrado durante varios años en un número elevado de muestras clínicas en China y Taiwán (Huang et al., 2015; Li et al., 2020).

En esta tesis doctoral, se han investigado distintas características, tanto fenotípicas como moleculares, de las cepas encontradas en las plantas de jamón curado. Así, se seleccionaron 12 cepas representantes de los tipos de secuencia mayoritarios y consideradas persistentes y no persistentes para determinar la variabilidad en genes de virulencia, la formación de biofilms y la presencia de genes de resistencia a

desinfectantes. La presencia de codones de parada prematuros (PMSCs) en las secuencias de algunos genes de virulencia de *L. monocytogenes*, como el codificante de la InlA y el del factor PrfA, se ha relacionado con cepas ampliamente distribuidas en industrias alimentarias (Chen et al., 2011). Por otro lado, algunas características de *L. monocytogenes* podrían contribuir a la persistencia de cepas del patógeno en el ambiente de procesado de alimentos, como la tolerancia a desinfectantes o la capacidad de adherirse a superficies de contacto y formar biofilms (Borucki et al., 2003; Lee et al., 2019; Lundén et al., 2000, 2003; Martínez-Suárez et al., 2016; Stoller et al., 2019). Sin embargo, otros autores no han encontrado evidencias de una mayor resistencia a desinfectantes o capacidad de adhesión y formación de biofilm en subtipos persistentes (Carpentier y Cerf, 2011; Ferreira et al., 2011; Jensen et al., 2008; Kabuki et al., 2004; Sereno et al., 2019). En este trabajo, no se ha podido establecer una conexión concreta, ya que se investigaron cepas que se consideraron persistentes, como la C002, en la que se detectaron genes de resistencia a desinfectantes, y la B007, en la que no se detectaron, pero ambas presentaron PMSCs en la InlA y fueron capaces de formar biofilm a 22 ºC. Por otro lado, cepas consideradas no persistentes, también presentaron PMSCs en la secuencia de la InlA (A001, 09, B001, C015, C021), genes de resistencia a desinfectantes (A001, C021) y fueron muy buenas formadoras de biofilm a 22 °C (A001). La única cepa 4b (ST1) aislada en este trabajo no presentó PMSCs en las secuencias de la InlA y el PrfA, ni genes de resistencia a desinfectantes, y fue de las cepas con menor capacidad de formación de biofilm a 22 ºC.

Hasta la fecha, se han identificado 21 mutaciones naturales que generan PMSCs en la secuencia del gen codificante de la InlA, que se relacionan con proteínas truncadas (Gelbíčová et al., 2015, 2016; Van Stelten et al., 2010). El gen *inlA* de *L. monocytogenes* codifica la internalina A, que tiene gran afinidad por los receptores de E-cadherina de células epiteliales humanas, por lo que es fundamental para la invasión del epitelio intestinal. En este trabajo, se secuenció el gen completo *inlA* de 12 cepas seleccionadas y se vio que seis de ellas, todas las pertenecientes al ST121, presentaron PMSC6, mientras que otra cepa, ST9, presentó PMSC19. El PMSC6 se describió por primera vez en un aislado de una mujer embarazada portadora sana en Francia (Olier et al., 2002) y ha sido detectado previamente en aislados procedentes de alimentos y su entorno y algunos casos clínicos de varios países, como Francia (Ragon et al., 2008), EE. UU. (Van Stelten et al., 2010), España (López-Alonso et al., 2020; Ortiz et al., 2016), Portugal (Ferreira da Silva et al., 2017; Ferreira et al., 2011), China (Su et al., 2019) o Chile (Bustamante et al.,

2020). El PMSC19 se describió por primera vez en una cepa de *L. monocytogenes* aislada de una listeriosis neonatal (Gelbíčová et al., 2015). En este trabajo, solo se detectó en una de las cepas 1/2c de las tres ST9 analizadas, lo que apunta a una mutación específica de cepa. De hecho, Ragon et al. (2008) discutieron acerca de la poca frecuencia e incluso inexistencia de la transmisión horizontal de alelos de *inlA* completos, por lo que las secuencias de este gen serían específicas de clon. Este tipo de mutación en la InlA ha sido menos identificada, aunque se ha encontrado en China, en 10 aislados también 1/2c (Su et al., 2019).

Casi todas las cepas de *L. monocytogenes* causantes de listeriosis presentan una InlA completa (Ragon et al., 2008). Jacquet et al. (2004) observaron que el 100, el 98 y el 93% de mujeres embarazadas con listeriosis, pacientes con infecciones del sistema nervioso central y pacientes con bacteriemia, respectivamente, expresaban una InlA completa y eran de serotipo 4b. Sin embargo, un porcentaje considerable de aislados de alimentos y plantas de procesado portan mutaciones que generan PMSCs (Jacquet et al., 2004; López-Alonso et al., 2020; Nightingale et al., 2005, 2008; Van Stelten et al., 2010). Un estudio realizado en Francia reveló la presencia de la InlA completa en el 65 y 96% de cepas procedentes de alimentos y cepas clínicas, respectivamente (Jacquet et al., 2004). En Italia, Tamburro et al. (2010) detectaron secuencias de InlA truncadas en el 89,0 y 85,7% de aislados de alimentos y ambiente de plantas de procesado, respectivamente, mientras que aparecía completa en el 93,8% de los aislados clínicos. Las cepas de *L. monocytogenes* portadoras de InlA truncada se han relacionado con infecciones asintomáticas (Olier et al., 2003) y diversos estudios afirman que este tipo de cepas presentan menor capacidad de adhesión e infección en las células intestinales humanas Caco-2 (Ferreira da Silva et al., 2017; Su et al., 2019). Nightingale et al. (2008) han observado incluso eficiencias de invasión significativamente reducidas en infecciones orales en cobayas, y afirman que cepas del patógeno de virulencia atenuada, ampliamente aisladas de alimentos y plantas de procesado, confieren inmunidad frente a posibles infecciones posteriores con cepas totalmente virulentas. Por otro lado, otros autores han detectado InlA con PMSCs, distintos a los tipos 6 y 19 observados en este trabajo, en cepas de *L. monocytogenes* virulentas (Roche et al., 2012). Recientemente, se ha publicado que el 20% de cepas aisladas en Brasil de alimentos de origen animal y afines presentaron PMSC19 en la secuencia de la InlA, y que dichas cepas mostraron mayor capacidad para adherirse e invadir células Caco-2 que las que no presentaban codones de parada (Medeiros et al., 2021). Como se ha descrito anteriormente, una cepa procedente

de una de las industrias investigadas presentó PMSC19, por lo que sería especialmente interesante investigar su capacidad de adhesión e invasión utilizando líneas celulares humanas, ya que los pocos datos bibliográficos existentes sobre esta mutación la relacionan con cepas virulentas.

Por último, tres de las cepas analizadas en este trabajo, presentaron a lo largo de su secuencia mutaciones que dieron lugar a diferentes aminoácidos. Aunque estos cambios no generaron PMSCs, estas variaciones en la cadena aminoacídica de la InlA podrían reducir la afinidad de la proteína por su receptor E-cadherina. En este sentido, Jensen et al. (2008) concluyeron que cepas alimentarias con mutaciones no sinónimas que no generaban PMSCs eran menos capaces de adherirse e invadir células Caco-2 que cepas clínicas. Aunque en el presente trabajo se ha realizado una primera aproximación a nivel génico, sería muy interesante como línea de futuro investigar la capacidad de estas cepas obtenidas en plantas de jamón curado para invadir líneas celulares intestinales humanas.

El factor principal de virulencia de *L. monocytogenes* PrfA es esencial para la expresión de un conjunto de genes de virulencia clave en la patogenicidad de la bacteria. Existen también mutaciones en la secuencia del PrfA que dan lugar a proteínas truncadas o no funcionales (Velge et al., 2007). En la presente tesis doctoral, se secuenció un fragmento del *prfA* que contiene dos tipos de mutaciones descritas por Roche et al. (2005), una supone una sustitución aminoacídica y la otra genera un PMSC. Las mutaciones detectadas en la secuencia del *prfA* no generaron cambios en la pauta de lectura ni dieron lugar a PMSCs, resultados que concuerdan con la baja incidencia de cepas de *L. monocytogenes* mutantes en PrfA en las industrias alimentarias (López-Alonso et al., 2020; Ortiz et al., 2016; Palma et al., 2017). Algunos autores han relacionado mutaciones en el PrfA con una pérdida o reducción de la virulencia mediante el uso de líneas celulares o modelos animales (López et al., 2013; Roche et al., 2012). Estas mutaciones también se han asociado con fenotipos de *L. monocytogenes* no hemolíticos, lo que se explicaría por el hecho de que el PrfA controla la expresión del gen *hly*, codificante de la proteína hemolítica LLO (López-Alonso et al., 2020; Maury et al., 2016, 2017).

Como se ha comentado previamente, la tolerancia a desinfectantes y la capacidad de formación de biofilms podría contribuir al establecimiento de cepas de *L. monocytogenes* en las industrias alimentarias y a su persistencia durante períodos prolongados de tiempo. La primera barrera para el control y la prevención de *L. monocytogenes* en la industria son las buenas prácticas de limpieza y desinfección. Los compuestos de amonio cuaternario, entre los que se incluye el cloruro de benzalconio

(CB), son los más utilizados en la desinfección de industrias alimentarias. A concentraciones adecuadas, estos compuestos son efectivos frente a *L. monocytogenes*, aunque a menudo se dan concentraciones de desinfectante subinhibitorias, principalmente en zonas de difícil acceso o como consecuencia de una limpieza incorrecta antes de la desinfección, que pueden desencadenar una tolerancia del patógeno que le permite crecer en presencia de concentraciones subletales del biocida (Cerf et al., 2010). En publicaciones recientes se concluye que la exposición repetida a concentraciones subinhibitorias de compuestos de amonio cuaternario permite a *L. monocytogenes* desarrollar tolerancia al CB (Noll et al., 2020; Yu et al., 2018). La tolerancia a los desinfectantes está mediada por factores genéticos codificantes de bombas de eflujo, que son proteínas transmembrana que expulsan y limitan la acumulación intracelular de agentes antimicrobianos a niveles subinhibitorios, de modo que la bacteria puede sobrevivir más tiempo (Piddock, 2006).

Dos de las seis bombas de eflujo asociadas a la tolerancia a CB están codificadas por el gen *qacH* y el casete *bcrABC*, ambos localizados en elementos genéticos móviles. El primero se encuentra en el transposón Tn*6188* (Müller et al., 2013) y el segundo en el plásmido pLM80 (Elhanafi et al., 2010), aunque pueden integrarse también en el genoma de *L. monocytogenes*. Müller et al. (2013) detectaron el gen *qacH* en 10 de 91 cepas de *L. monocytogenes*, mientras que el *bcrABC* se registró en 70 de 71 cepas resistentes a CB, aisladas principalmente del ambiente de industrias alimentarias (Dutta et al., 2013). El 14,9% de aislados de *L. monocytogenes* de nueve plantas noruegas de procesado de carne y salmón presentaron los genes *qacH* y/o *bcrABC* (Møretrø et al., 2017), registrándose este último en el 41,5% de aislados procedentes de alimentos e industrias de Canadá (Cooper et al., 2021), y en el 22,1% de aislados procedentes de una industria cárnica de Eslovaquia (Minarovičová et. al, 2018). En los cuatro casos, la concentración mínima inhibitoria (CMI) para el CB fue mayor en las cepas que presentaban los genes de resistencia. Según Harrand et al. (2020), de un total de 40 aislados de *L. monocytogenes* de una planta de procesado de salmón, 36 presentaron el casete *bcrABC* y uno el gen *qacH*. En un estudio realizado en China, Jiang et al. (2016) detectaron que 13 aislados de alimentos de un total de 59 eran resistentes a CB, dos de los cuales portaban el casete *bcrABC* y ninguno el *qacH*. Ortiz et al. (2016) detectaron el gen *qacH* en cuatro de cinco cepas de serotipo 1/2a aisladas de dos plantas de procesado de cerdo ibérico y resistentes a CB, mientras que ninguna portaba el casete *bcrABC*. En otro trabajo, el 12,3% de aislados de origen alimentario y clínico de Suiza fue tolerante a CB, portando el 62,5 y

16,7% de ellos el gen *qacH* y el casete *bcrABC*, respectivamente, mientras que dicha tolerancia se registró en un 10,6% de aislados de origen alimentario de Finlandia, con un 52,4% de estas cepas portadoras del *qacH* (Meier et al., 2017). La mayoría de estas cepas portadoras de genes de resistencia analizadas por Meier et al. (2017) fueron ST121 y ST9, relacionándose una vez más la presencia de genes de resistencia a desinfectantes con cepas comúnmente aisladas de alimentos e industrias alimentarias. En el trabajo desarrollado en esta tesis doctoral, no se detectó el casete *bcrABC* de resistencia a CB en ninguna cepa, pero tres de las 12 cepas analizadas (25,0%) portaron el transposón Tn*6188*, ya que se amplificó por PCR el gen *qacH*. Todas ellas correspondían al serotipo 1/2a y al ST121. Una de estas cepas fue considerada persistente según el criterio establecido y se aisló de diferentes puntos de una de las tres plantas de procesado durante la producción y tras la limpieza y desinfección, tanto de superficies de contacto como de zonas de no contacto. Con vistas a futuro y con el fin de obtener más información al respecto, sería interesante realizar un análisis fenotípico de la posible tolerancia de las cepas a CB, para ver si su CMI es mayor para las cepas portadoras de genes de resistencia. También se podría llevar a cabo un análisis transcriptómico para determinar si la expresión del gen de resistencia *qacH* se induce en presencia del desinfectante, ya que algunos autores han observado que la expresión de genes *bcrABC* aumenta en presencia de CB (Dutta et al., 2013; Elhanafi et al., 2010; Minarovičová et al., 2018).

En la tolerancia microbiana a desinfectantes, además de la transferencia horizontal de genes de resistencia desde una cepa resistente a una población sensible, intervienen también una serie de alteraciones fenotípicas, como la transición a un estado viable no cultivable (Noll et al., 2020), cambios en la composición de las superficie celular que suponen una reducción de la permeabilidad de las membranas del patógeno a los compuestos (Duze et al., 2021; Martínez-Suárez et al., 2016) o la formación de biofilms. Los biofilms están formados por comunidades microbianas complejas que se adhieren a superficies bióticas o abióticas mediante la secreción de una matriz extracelular, que supone aproximadamente el 90% del biofilm (Colagiorgi et al., 2016, 2017; Ripollés-Ávila et al., 2018). Se forman como una respuesta adaptativa y proporcionan a las bacterias una serie de ventajas como transferencia de material genético o protección frente a desecación, antibióticos, bioicidas, etc. (Mazaheri et al., 2021). El problema de la formación de biofilms en las industrias alimentarias reside en que las bacterias resisten mejor a los tratamientos de limpieza y desinfección debido a que la matriz extracelular del biofilm actúa como barrera creando un microambiente y limitando la difusión de

compuestos antimicrobianos, lo que contribuye a la aparición de cepas tolerantes que pueden sobrevivir en presencia de concentraciones de desinfectante cada vez mayores (Bridier et al., 2011; Martínez-Suárez et al., 2016; Ruiz-Bolivar et al., 2008; Tezel y Pavlostathis, 2015). Por tanto, la formación de biofilms podría estar relacionada con la persistencia bacteriana en las superficies y ambiente de las industrias, con la subsecuente posibilidad de contaminación de productos. *L. monocytogenes* tiene la capacidad de adherirse y formar biofilms en las superficies, sobre todo donde se acumulan residuos de alimento (Colagiorgi et al., 2017). En este trabajo, se analizó la capacidad de formación de biofilms de 12 cepas de *L. monocytogenes* procedentes de las tres plantas de jamón curado durante 7 días a 8 ºC y durante 4 días a 22 ºC. Ninguna de las cepas fue capaz de formar biofilm a 8 ºC, mientras que todas excepto una lo formaron a 22 ºC. Estos resultados concuerdan con los obtenidos por Stoller et al. (2019) y Lee et al. (2019), en los que se registró el doble de masa de biofilm tras 96 h a 22 ºC en comparación con una incubación de 168 h a 8 ºC y cinco veces más tras 24 h a 37 ºC en comparación con una incubación a 10 ºC, respectivamente. Sin embargo, el hecho de que *L. monocytogenes* forme biofilms en las industrias alimentarias, donde pueden darse temperaturas en torno a 8-10 ºC, conduce a pensar que hay una serie de factores ambientales que promueven su formación. En el caso de las industrias de jamón curado podría deberse a la presencia de sal y concentraciones insuficientes de desinfectantes, que, junto a las condiciones de frío, podrían estar seleccionando subtipos de *L. monocytogenes* con características adaptativas que les ayuden a persistir (Lee et al., 2019; Maury et al., 2019; Pan et al., 2010; Tasara y Stephan, 2007). De acuerdo con nuestros resultados, la mayoría de cepas ST121 (serotipo 1/2a), a excepción de una, formaron biofilm a 22 ºC, así como la cepa ST9 (serotipo 1/2c), lo que concuerda con los resultados obtenidos por otros autores, que afirman que las cepas del linaje II son mejores formadoras de biofilm que las del linaje I, y principalmente las cepas 1/2a (Borucki et al., 2003; Ortiz, 2016; Pan et al., 2010). Las cepas ST121 mejores formadoras de biofilm a 22 ºC presentaron mutaciones en la secuencia de InlA. Franciosa et al. (2009) también observaron que las cepas con InlA truncada presentaban mayor capacidad de formar biofilm e incluso hipotetizaron que dicha proteína liberada al medio podría formar parte de la matriz extracelular del biofilm. Por último, algunos autores han afirmado que la presencia de concentraciones subinhibitorias de desinfectantes puede promover la formación de biofilm en *L. monocytogenes* (Maury et al., 2019). Por ello, sería interesante investigar la capacidad de formación de biofilm de estas cepas en presencia de distintas concentraciones de desinfectantes.

En definitiva, el control de *L. monocytogenes* en la industria productora de jamón es un elemento clave para asegurar la seguridad y la calidad del producto. Sin embargo, no es tarea fácil ya que, como se ha visto, los procesos de limpieza y desinfección no eliminan todas las bacterias, y aquellas que son capaces de sobrevivir podrían llegar a establecerse, persistir y representar una fuente de contaminación cruzada. Por ello, un estudio como el realizado en la presente tesis doctoral podría resultar muy útil a la industria jamonera. Tener un mayor conocimiento acerca de genes de virulencia de las cepas que se aíslan de las distintas empresas, a través, por ejemplo, del análisis de la InlA, podría permitir a los productores hacer una estimación del riesgo, basada no solo en función de la presencia y de los niveles de contaminación bacteriana, sino también en la virulencia potencial de dichas cepas. Por otro lado, la capacidad de determinar marcadores genéticos de resistencia a desinfectantes, así como el conocimiento de la formación de biofilm, podría servir como herramienta para evaluar posibles fuentes de contaminación persistentes en el ambiente de procesado de jamón curado, permitiendo desarrollar estrategias específicas de control para mejorar la eficacia de las operaciones de desinfección.

2. Tratamientos de inactivación de *L. monocytogenes* en jamón curado

Teniendo en cuenta la dificultad en la erradicación de *L. monocytogenes* y la importancia de su presencia en distintos productos cárnicos RTE envasados o loncheados, que puede dar lugar a casos de listeriosis, así como problemas de comercialización, se han investigado distintos tratamientos de control.

Las altas presiones hidrostáticas (APH) es la tecnología no térmica más utilizada industrialmente para reducir los niveles de microorganismos patógenos y alterantes en los alimentos, promoviendo la conservación y extendiendo la vida útil de los productos. El número de equipos de APH está creciendo exponencialmente y los productos cárnicos representan del 25 al 30% del total de los alimentos presurizados del mercado (Jung y Tonello-Samson, 2018), estimándose que durante 2019 se procesaron por APH unas 400 000 toneladas de productos cárnicos (Bolumar et al., 2021). Esta tecnología ha sido ampliamente aceptada por parte de los consumidores debido a que los cambios sensoriales que perciben en los productos presurizados son mínimos (García-Gimeno y Posada, 2020).

Por ello, y siempre utilizando jamón curado como matriz alimentaria, en esta tesis doctoral se han ensayado métodos de control de *L. monocytogenes* basados en APH,

aplicadas de forma individual o en combinación con otros antimicrobianos, evaluando, además, los efectos de los tratamientos sobre las características fisicoquímicas, la textura y el color del jamón, ya que es importante garantizar tanto la seguridad como la calidad del producto al consumidor.

2.1. Tratamientos de altas presiones en jamones deshuesados enteros

En un primer trabajo (Publicación 2.1), se analizó el efecto de las altas presiones sobre *L. monocytogenes* en jamones curados deshuesados enteros, así como su impacto en las propiedades del producto. Los jamones se inocularon con un cóctel de 4 cepas de *L. monocytogenes* de los serotipos 1/2a, 1/2b, 1/2c y 4b, en superficie y en interior, se presurizaron a 450 o 600 MPa durante 10 o 5 min, respectivamente, y se mantuvieron durante 60 días a 4 o 12 ºC. En los días 1, 30 y 60 de ensayo, se tomaron muestras de las zonas inoculadas y no inoculadas para determinar los niveles de *L. monocytogenes* y para analizar el pH, la a_w, el contenido de humedad y la concentración de cloruros y nitritos, respectivamente.

Un día después de la presurización, el tratamiento moderado de 450 MPa durante 10 min consiguió reducciones de 0,9 y 1,4 unidades logarítmicas en superficie e interior, respectivamente, mientras que con el tratamiento de 600 MPa durante 5 min se alcanzaron reducciones de 1,9 y 2,9 unidades. Tras 60 días de almacenamiento, los niveles del patógeno disminuyeron con el tratamiento a 450 MPa durante 10 min 1,2 y 0,7 unidades logarítmicas en la superficie y 2,0 y 1,7 en el interior, a 4 y 12 ºC, respectivamente. Con el tratamiento de APH más intenso (600 MPa/5 min), los recuentos de *L. monocytogenes* en la superficie del jamón mantenido a 4 y 12 ºC fueron 2,1 y 0,8 unidades logarítmicas menores que en el jamón no tratado, respectivamente, siendo estas diferencias mucho mayores en el interior del producto, en torno a 3,4 unidades a las dos temperaturas ensayadas. De Alba et al. (2015) aplicaron un tratamiento de 450 MPa durante 10 min en jamón curado loncheado y observaron reducciones de *L. monocytogenes* de alrededor de 0,6 unidades logarítmicas tanto a las 24 horas como tras 60 días de conservación a 8 ºC, resultados similares a los obtenidos en este trabajo en las muestras de superficie cuando los jamones deshuesados fueron sometidos a ese mismo tratamiento. También Bover-Cid et al. (2011) obtuvieron reducciones bajas, de 0,32 unidades, en jamón curado con un tratamiento de APH similar. Cuando se aplicaron presurizaciones más intensas, de 600 MPa durante 6 min, se lograron reducciones de *L. monocytogenes* mayores en jamón curado, aunque la ausencia en 25 g solo se consiguió en productos cárnicos con mayor a_w

(Jofré et al., 2009b). Stollewerk et al. (2012) comprobaron la ausencia de *L. monocytogenes* en jamón curado ahumado a partir del día 14 tras un tratamiento de 600 MPa durante 5 min y no detectaron la recuperación del patógeno durante el almacenamiento, incluso en condiciones de aumento de temperatura.

Los recuentos de *L. monocytogenes* disminuyeron en la superficie de los jamones control (no presurizados) durante los 60 días de almacenamiento. En un estudio reciente, Serra-Castelló et al. (2020) concluyeron que la baja a_w del jamón curado loncheado resultó listericida frente al mismo cóctel de cepas utilizado en este trabajo, y propusieron el control de este parámetro, junto con almacenamiento en condiciones adecuadas de temperatura, como una medida para garantizar la seguridad de este producto y cumplir con los requisitos legales de comercialización. En base a lo observado en el presente trabajo, controlar la temperatura y la a_w no sería suficiente en caso de que se produjera una contaminación en el interior del jamón durante el deshuesado, ya que los niveles de *L. monocytogenes* inoculada en el interior de jamones no presurizados se mantuvieron constantes durante los dos meses de almacenamiento a 4 y 12 ºC, por lo que en este caso sería recomendable aplicar un tratamiento de higienización. Los tratamientos de presurización ensayados resultaron mucho más efectivos frente a *L. monocytogenes* en el interior de los jamones deshuesados, hecho atribuido a los mayores valores de a_w respecto a los registrados en la superficie del producto, significativamente inferiores. Se ha descrito que la a_w de los alimentos juega un papel muy importante en la eficacia de las APH, ya que la resistencia de los microorganismos al tratamiento aumenta a medida que disminuye el valor de este parámetro (Campus, 2010; Patterson, 2005), como se discutirá en el siguiente apartado.

En cuanto al análisis fisicoquímico, se detectaron modificaciones mínimas en los jamones deshuesados enteros presurizados con respecto a los jamones control. Los valores de pH de las muestras de superficie e interior de los jamones tratados y control y almacenados durante 60 días a 4 y 12 ºC oscilaron entre 5,42 y 6,03. Aunque se observó un ligero descenso en el pH de las muestras del interior del jamón un día después del tratamiento, los valores tendieron a estabilizarse a lo largo del almacenamiento y, por lo general, no se detectaron diferencias significativas en el pH de los jamones deshuesados como consecuencia de la presurización. De Alba et al. (2013a) registraron un ascenso mínimo en los valores de pH en jamón curado sometido a APH, aunque también tendieron a estabilizarse. Si bien apenas se ha investigado el efecto de las APH sobre el pH del jamón curado, sí se ha analizado el efecto del pH en la eficacia de las APH y se ha

concluido que valores bajos contribuyen a mejorar la efectividad de la presurización. En este sentido, Serra-Castelló et al. (2020) atribuyeron mejores resultados en la inactivación de *L. monocytogenes* en jamón Ibérico en comparación con jamón Serrano al pH más bajo, entre otros factores, por lo que el pH ligeramente ácido del jamón curado podría suponer un factor intrínseco que favorece la inactivación de *L. monocytogenes* por APH.

En el caso de la a_w, se observaron diferencias significativas en función del tratamiento, la zona del jamón y el tiempo de almacenamiento, con una tendencia a la estabilización. En general, los valores de a_w, de 0,858-0,887 y 0,875-0,894 en superficie e interior, respectivamente, aumentaron durante los dos meses de ensayo, en muestras presurizadas y no presurizadas y a las dos temperaturas investigadas. Estos resultados coinciden con los observados por De Alba et al. (2012) y por Serra et al. (2007) en jamón curado loncheado y jamones enteros con tiempos cortos de maduración, respectivamente. También se observaron diferencias significativas en el contenido medio en humedad de los jamones, de 46,1%, en función de la zona del jamón y el tiempo de almacenamiento, aunque tras 60 días a 4 y 12 ºC, no se detectaron diferencias en el contenido de humedad de los jamones presurizados y no presurizados. Las altas presiones tampoco modificaron el contenido de humedad de salchichas curadas (Pietrasik et al., 2017), jamones curados reestructurados (Fulladosa et al., 2009) o jamón curado loncheado envasado al vacío (Clariana et al., 2011).

Por lo general, la presurización no produjo cambios en el contenido de nitritos, cuyo valor medio fue de 2,68 y 2,85 mg/kg en superficie e interior, respectivamente. Del mismo modo, solo algunas muestras presentaron ligeras diferencias en el contenido de NaCl tras el tratamiento, atribuidas a la variación inter-muestra. La concentración de sal fue de 3,1% en superficie y de 4,2% en interior. Clariana et al. (2011) tampoco registraron cambios en el contenido de nitritos ni de sal como consecuencia de presurizaciones a 600 MPa durante 6 min en jamón curado loncheado. Se ha observado que el efecto antimicrobiano de las APH aumenta en presencia de nitritos, aunque sea en bajas concentraciones (De Alba et al., 2013b), lo que hace que el jamón curado deshuesado sea un buen producto a la hora de aplicar este tipo de tratamientos. La mayor concentración de nitritos en las muestras de interior registrada en este trabajo podría haber contribuido a potenciar el efecto listericida de la APH. De manera similar, Morales et al. (2006) comprobaron reducciones de *L. monocytogenes* mayores en jamón Ibérico que en Serrano al aplicar 450 MPa durante 10 min, lo que se atribuyó, en parte, a las diferencias en el contenido de sal. Sin embargo, el efecto baroprotector no deseado, ligado a la presencia de NaCl y, por

tanto, a la a_w, podría compensarse ya que estas características dificultarían la recuperación del patógeno tras la presurización y durante el almacenamiento del jamón.

Hasta la fecha no se ha informado de ningún brote de listeriosis asociado al consumo de jamón curado. No obstante, la presencia global de *L. monocytogenes* en muestras comerciales de jamón curado varía desde la no detección hasta una prevalencia de incluso el 12% (Serra-Castelló et al., 2020). Además de contribuir a la notificación de algunas alarmas sanitarias, este hecho podría perjudicar la exportación de jamón curado español, que ha crecido notablemente en los últimos años (Mercasa, 2019; Rodríguez, 2018). En los países miembros de la UE, principales destinatarios del jamón curado español, el reglamento establece un máximo de 100 UFC/g de *L. monocytogenes* siempre y cuando el producto no esté destinado para lactantes o fines médicos especiales y no favorezca el crecimiento de la bacteria en más de 100 UFC/g al final de la vida útil. Según el mismo reglamento, el jamón curado, que presenta valores de a_w iguales o inferiores a 0,92, se describiría como un producto RTE incapaz de soportar el crecimiento de *L. monocytogenes* (EC, 2005). Algunos países importadores de jamón curado de origen español, como Australia, siguen una política de tolerancia similar, mientras que otros, como EE. UU., se rigen por una legislación más estricta de tolerancia cero o ausencia en 25 g. Por un lado, en este trabajo, y de acuerdo con los resultados de Montiel et al. (2020), se ha visto que *L. monocytogenes* inoculada en la superficie de jamones deshuesados enteros disminuye con el tiempo de almacenamiento, y, por otro lado, las presurizaciones a 600 MPa durante 5 min consiguieron reducciones en el interior del jamón de en torno a 3,4 unidades logarítmicas, tanto a 4 como a 12 ºC. Estas reducciones permiten superar en más de una unidad el objetivo europeo de seguridad alimentaria (FSO), así como el criterio establecido por el cual un tratamiento se considera listericida en jamón curado, de 2,0 y 2,4 reducciones logarítmicas, respectivamente, en el momento del consumo (EC, 2005; Hoz et al., 2008). Esto, sumado a la baja significación de los cambios observados en las propiedades fisicoquímicas, permite concluir que el tratamiento de APH de 600 MPa durante 5 min sería una buena alternativa para inactivar *L. monocytogenes* e impedir su recuperación durante 60 días en jamones deshuesados enteros almacenados bajo condiciones de refrigeración estrictas (4 ºC) o en condiciones de abuso de temperatura (12 ºC) en caso de que se produjera una contaminación durante el postprocesado.

2.2. Tratamientos de altas presiones en jamón curado loncheado con distinta a_w

En vista de la influencia de la a_w en la efectividad de los tratamientos de APH en los alimentos en general, y en jamón curado en particular, se planteó un trabajo (Publicación 3.2) para corroborar su impacto en la inactivación de *L. monocytogenes* en jamón curado con distinta a_w y tratado por altas presiones. Para ello, se modificó la a_w de lonchas de jamón curado (a_w inicial = 0,94) sometiéndolas a un equilibrio vapor-líquido con sales de cloruro saturadas hasta alcanzar valores de 0,92, 0,88 y 0,84. Posteriormente, se inocularon de forma individual con *L. monocytogenes* S4-2 (serotipo 1/2b) y S12-1 (serotipo 1/2c), seleccionadas por ser la más y la menos resistente a las altas presiones, respectivamente, de las cuatro cepas con las que se ha trabajado en esta tesis. Las muestras se presurizaron a 450 y 600 MPa durante 10 y 5 min, respectivamente y se almacenaron a 4 ºC durante un mes. Los niveles de *L. monocytogenes* se determinaron en los días 1, 15 y 30 tras la presurización, mientras que el contenido en NaCl y nitritos tras la modificación de la a_w y el pH y el número total de células viables (TVC) tras 1, 15 y 30 días de la aplicación de la APH se determinó en muestras no inoculadas.

A las 24 horas de la presurización, los niveles de las cepas S4-2 y S12-1 disminuyeron aproximadamente 1,0 y 1,5 unidades logarítmicas con el tratamiento de 450 MPa durante 10 min, respectivamente, en jamón curado con distinta a_w. El tratamiento más intenso (600 MPa/5 min) dio lugar a reducciones mayores, de 2,5 y 2,8 log UFC/g para las cepas S4-2 y S12-1, respectivamente. La disminución de la a_w en el jamón curado ejerció un efecto baroprotector sobre ambas cepas. Así, con a_w de 0,88 y el tratamiento más intenso, las reducciones registradas fueron 1,0 y 1,6 unidades, respectivamente, es decir, más de una unidad logarítmica inferiores a las obtenidas con una a_w de 0,92. Durante los 30 días de almacenamiento a 4 ºC, los niveles de *L. monocytogenes* se mantuvieron sin cambios en las muestras no presurizadas. A los 30 días, la cepa S4-2, más resistente a las APH, presentó recuentos de 1,4 y 3,0 unidades logarítmicas inferiores a los del jamón control en los jamones con a_w 0,92 presurizados a 450 MPa/10 min y 600 MPa/5 min, respectivamente, sin registrarse variaciones en muestras con a_w inferiores. Para la S12-1, las diferencias con respecto a las muestras control tras 30 días fueron de 2,1 y 3,4 unidades logarítmicas para los tratamientos a 450 y 600 MPa, respectivamente, en las muestras con la a_w más alta. En jamón con a_w de 0,88 se registraron reducciones de 2,0 y 1,7 unidades para el tratamiento moderado y el intenso,

respectivamente, pero con la a_w más baja, los niveles de *L. monocytogenes* se mantuvieron constantes durante el almacenamiento.

Este efecto baroprotector fue observado previamente en lonchas de jamón curado (Bover-Cid et al., 2015), con reducciones de *L. monocytogenes* entre 2,2 y 6,8 unidades logarítmicas a medida que se aumentaba la a_w del producto mediante adición de agua, desde valores de 0,860 a 0,960. Del mismo modo, la eficacia de un tratamiento de APH de 600 MPa durante 5 min frente a *L. monocytogenes* disminuyó en muestras de jamón curado con valores más bajos de a_w, con reducciones de 3,9 y 1,9 unidades para valores de 0,92 y 0,88, respectivamente (Hereu et al., 2012). Morales et al. (2006) observaron una mayor inactivación de *L. monocytogenes* a 450 MPa durante 10 min en jamón Ibérico (a_w = 0,90) que en jamón Serrano (a_w = 0,88). Esta disminución en la eficacia de las altas presiones como consecuencia de la baja a_w también explicaría las diferencias descritas en el apartado anterior entre el interior y la superficie de jamones deshuesados enteros presurizados. En la Publicación 3.1 de esta tesis, las cepas de *L. monocytogenes* S2 (serotipo 1/2a) y S7-2 (serotipo 4b) mostraron una sensibilidad moderada a las APH (450 MPa/10 min y 600 MPa/5 min), con reducciones del patógeno inferiores a 1,5 unidades logarítmicas, lo que se atribuyó a la baja a_w del jamón curado loncheado (a_w = 0,88). Estos resultados son similares a los obtenidos en este trabajo en muestras de jamón con la misma a_w.

En cuanto a los TVC, a las 24 horas de la presurización, los dos tratamientos (450 MPa/10 min y 600 MPa/5 min) redujeron los niveles de TVC entre una y dos unidades logarítmicas para todos los valores de a_w. Durante los 30 días de almacenamiento, solo se registró un incremento en TVC en las muestras control con a_w de 0,92. Por el contrario, no se observó crecimiento de microorganismos totales en las muestras no presurizadas con a_w de 0,88 y 0,84, ni en ninguna de las muestras presurizadas, en las que los recuentos se mantuvieron por debajo de 10^6 UFC/g tras 30 días de almacenamiento a 4 ºC, independientemente de la a_w. En ninguno de los casos se alcanzaron niveles de TVC de 10^7 UFC/g, que generalmente se asocian a alteraciones del alimento. Piras et al. (2016) registraron niveles de TVC entre 6 y 7 unidades logarítmicas en jamón curado envasado al vacío almacenado a 2 y 8 ºC durante 63 días. Con tratamientos de APH de 600 MPa durante 6 min en jamón curado loncheado (Garriga et al., 2004), los TVC disminuyeron al menos 2 unidades logarítmicas, y se mantuvieron por debajo de 10^3 UFC/g durante 120 días de almacenamiento a 4 ºC. Sin embargo, también se han observado aumentos en los niveles de TVC durante el almacenamiento de jamón curado presurizado tras la

inactivación por el tratamiento (Clariana et al., 2011). Respecto al efecto de la presurización en los distintos grupos microbianos, con tratamiento de 600 MPa durante 6 min las reducciones observadas para mesófilos aerobios, psicrotrofos, bacterias lácticas, micrococos, y mohos y levaduras fueron de 1,63, 1,71, 0,44, 1,15 y 1,95 unidades logarítmicas, respectivamente (Martínez-Onandi et al., 2017). En esta tesis, los valores de pH en muestras tratadas y no tratadas, con distintas a_w y en distintos tiempos de análisis oscilaron entre 5,82 y 6,14. Tras 30 días de almacenamiento a 4 ºC, no se registraron diferencias significativas de pH entre muestras control y presurizadas, excepto en el caso de jamón con a_w 0,84, donde las diferencias fueron reducidas, de media unidad como máximo. A lo largo de la refrigeración, se observó un ligero descenso en los valores de pH en muestras con a_w bajas, de 0,88 y 0,84. En cualquier caso, los cambios de pH detectados en función de la a_w fueron mínimos, al igual que las diferencias registradas como consecuencia de la presurización, tal y como se vio en la discusión del apartado anterior. La modificación de la a_w tampoco afectó al contenido de nitritos del jamón, con valores en torno a 4,0 mg/kg. Como era de esperar, la reducción de la a_w produjo un aumento en el porcentaje de NaCl (peso/peso), pero estas diferencias no fueron significativas.

Este trabajo resalta la importancia de la matriz alimentaria en la efectividad de las altas presiones frente a *L. monocytogenes*. En el caso del jamón curado, la baja a_w, característica de este producto, podría comprometer la sensibilidad del patógeno a los tratamientos de presurización. No obstante, y en base a los resultados obtenidos en esta tesis, la propia a_w baja impidió la recuperación y, consecuentemente, el crecimiento de *L. monocytogenes* en jamón curado loncheado.

2.3. Tratamientos combinados de altas presiones y enterocinas o timol en jamón curado loncheado

Si bien las altas presiones han demostrado su utilidad para inactivar *L. monocytogenes* en jamón curado, la baja a_w del producto podría reducir la eficacia del tratamiento. Por otra parte, la industria alimentaria busca aplicar tratamientos menos intensos sin que se comprometa su efectividad. Según la teoría de obstáculos de Leistner (2000), la combinación de APH con otras estrategias o agentes antimicrobianos permitiría reducir la intensidad de los tratamientos individuales y conseguir efectos sinérgicos, impidiendo la recuperación y/o el crecimiento de las células supervivientes a la presurización (Teixeira et al., 2018). En este sentido, en la presente tesis doctoral, se llevó

a cabo otro trabajo (Publicación 2.2) en el que se investigó el efecto anti-*Listeria* en jamón curado loncheado de un tratamiento moderado de altas presiones, individualmente y en combinación con enterocinas A y B o timol, así como su impacto en el número total de células viables y algunas características fisicoquímicas del producto. Las lonchas de jamón curado se inocularon con el cóctel de *L. monocytogenes* descrito en el apartado anterior. En la superficie de las lonchas se añadió un extracto de enterocinas A y B o timol a concentración de 1054 UA/g y 1,25 mg/g, respectivamente, y se aplicó un tratamiento de APH a 450 MPa durante 5 min. Las muestras se conservaron durante 30 días a 4 o 12 ºC, período durante el cual se realizaron análisis microbiológicos de *L. monocytogenes* y TVC en muestras inoculadas y no inoculadas, respectivamente, en los días 1, 7, 15 y 30, así como determinaciones de pH, a_w, color y textura en los días 1, 15 y 30 en muestras no inoculadas.

Se ha demostrado que el uso de bacteriocinas como agentes antimicrobianos en alimentos es una buena estrategia para controlar el crecimiento de patógenos en general y de *L. monocytogenes* en particular. En este trabajo, se preparó un extracto de enterocinas A y B a partir de un cultivo de *E. faecium* INIA TAB7 según lo descrito por Garriga et al. (2002). Un día después de su aplicación, las enterocinas A y B resultaron el tratamiento más eficaz frente a *L. monocytogenes*, con reducciones entre 2,5 y 3,0 unidades logarítmicas a 4 y 12 ºC, aunque su aplicación no impidió la recuperación del patógeno a partir del día 14 de almacenamiento a ambas temperaturas. Cuando se ensayaron las enterocinas A y B (Publicación 3.3) en jamón curado loncheado inoculado de forma individual con dos cepas de *L. monocytogenes* (S2, serotipo 1/2a, y S7-2, serotipo 4b), las reducciones alcanzadas fueron menores, en torno a la media unidad logarítmica para las dos cepas, tanto inmediatamente después de la aplicación del tratamiento, como tras 30 días a 4 o 20 ºC. Las diferencias en la eficacia de las enterocinas A y B observadas entre ambos trabajos podría deberse a una sensibilidad diferente por parte de las cepas de *L. monocytogenes* ensayadas. Buncic et al. (2001) y Moorhead y Dykes (2003) también registraron diferencias en el comportamiento de cepas de *L. monocytogenes* de distinto serotipo en presencia de bacteriocinas. Otras bacteriocinas, como las enterocinas L50A y L50B a 400 UA/mL evitaron el crecimiento del patógeno en jamón durante al menos 10 semanas a 8 ºC y 30 días a 15 ºC (Du et al., 2017). Asimismo, la nisina aplicada en la superficie de lonchas de jamón curado ocasionó reducciones inmediatas en la población de *L. monocytogenes*, mientras que su aplicación mediante envase activo no mostró unos efectos tan evidentes, aunque sí disminuyó la supervivencia del patógeno durante el

almacenamiento (Hereu et al., 2012). Mediante la aplicación individual de distintas bacteriocinas en jamón curado, se alcanzaron reducciones de 0,1 a 1,5 unidades logarítmicas de *L. monocytogenes*, siendo la pediocina la más efectiva y la nisina Z la menos (De Alba, 2014).

La inactivación obtenida al aplicar 450 MPa durante 10 min en jamón curado loncheado fue moderada, debido seguramente a la baja a_w del producto y a la menor intensidad del tratamiento. Estos resultados fueron semejantes a los discutidos en el apartado anterior para tratamientos similares. No obstante, su combinación con las enterocinas A y B mostró un efecto anti-*Listeria* sinérgico, con reducciones del patógeno en torno a 5,0 unidades logarítmicas, e impidió su recuperación durante todo el tiempo de estudio tanto a 4 como a 12 ºC. Se ha demostrado la eficacia de la combinación de APH y bacteriocinas frente a *L. monocytogenes* en distintos productos cárnicos, detectándose, por ejemplo, una mayor sensibilidad del patógeno al combinar APH y nisina en jamón cocido (Aymerich et al., 2005), un aumento de la vida útil en jamón cocido loncheado con un tratamiento de APH y enterocina LM-2 (Liu et al., 2012), la inhibición del crecimiento del patógeno mediante APH y enterocinas A y B en jamón cocido refrigerado (Marcos et al., 2008), recuentos menores a una unidad logarítmica con APH y enterocinas A y B en embutidos fermentados a temperatura ambiente (Jofré et al., 2009a), un efecto anti-*Listeria* sinérgico con APH combinadas con distintas bacteriocinas en *carpaccio* de ternera, como nisina Z, enterocinas A y B o pediocina PA-1 (De Alba, 2014), así como recuentos por debajo de 100 UFC/g durante 61 días a 4 ºC en un modelo de carne tratado con APH y sakacina K, enterocinas A y B o pediocina AcH (Garriga et al., 2002).

Con respecto a la combinación de APH y bacteriocinas en jamón curado, Jofré et al. (2008) comprobaron la ausencia de *L. monocytogenes* con un tratamiento de APH (600 MPa/5 min) con enterocinas A y B producidas por *E. faecium* CTC492, sakacina K producida por *L. sakei* CTC494 o nisina comercial, todas ellas a una concentración de 1280 UA/g, durante 120 días incluso en condiciones de abuso de temperatura. Hereu et al. (2012) concluyeron que la aplicación directa de nisina (200 UA/cm^2) incrementó la inactivación de *L. monocytogenes* inducida por la alta presión (600 MPa/5 min), siendo mayor en muestras de jamón con a_w más alta. De acuerdo con los resultados obtenidos en esta tesis, la combinación de APH a 450 MPa durante 10 min y enterocinas A y B consiguió reducciones mayores de *L. monocytogenes* que tratamientos de presurización más intensos (600 MPa/5 min) aplicados de forma individual en la superficie de jamones deshuesados, según lo descrito en el apartado anterior.

Cuando se investigó el efecto antimicrobiano de tres aceites esenciales (AEs, timol, carvacrol y cinamaldehído), se comprobó la misma CMI, 1,25 mg/mL, frente a *L. monocytogenes*, con una capacidad antimicrobiana similar en jamón curado, seleccionándose el timol para su combinación con un tratamiento de alta presión. Con la aplicación individual de timol se consiguieron reducciones moderadas de *L. monocytogenes*. En carne de vacuno cocinada al vacío, Gouveia et al. (2017) observaron una disminución de los niveles de *L. monocytogenes* con aceite esencial de tomillo (con un 7,5% de timol) a 2 ºC. Sin embargo, al aumentar la temperatura, los recuentos del patógeno se incrementaron a partir del séptimo día. Los niveles de *L. monocytogenes* disminuyeron en carne de vacuno marinada con salsa teriyaki a la que se añadió timol o carvacrol al 0,5%, y no se detectó tras siete días a 4 ºC (Moon et al., 2017). En el presente trabajo, la aplicación de timol en jamón curado loncheado no fue eficaz, posiblemente debido a una distribución poco homogénea como consecuencia de la afinidad del AE por otros compuestos hidrófobos, como las grasas (Pesavento et al., 2015; Shah et al., 2012). En combinación con altas presiones, los recuentos de *L. monocytogenes* se mantuvieron 1,30 y 1,45 unidades por debajo de los observados en las muestras control tras 30 días a 4 y 12 ºC, respectivamente. Con una concentración más alta seguramente podría conseguirse un efecto antibacteriano mayor, aunque superaría el umbral de aceptabilidad del sabor del producto (Pesavento et al., 2015). De hecho, la aplicación de timol a 1,25 mg/g afectó al olor del jamón curado. La combinación de AEs y alta presión frente a *L. monocytogenes* se ha investigado en productos no cárnicos, como queso fresco, en el que la combinación de APH con extracto de tomillo aceleró la cinética de inactivación del patógeno (Bleoancă et al., 2016). La combinación de APH (600 MPa/5 min) y aceites esenciales de menta en una bebida de yogur permitió disminuir la intensidad en 100-300 MPa y/o el tiempo en 210 s para conseguir reducciones de *L. monocytogenes* similares a las obtenidas con el tratamiento individual de APH (Evrendilek y Balasubramanian, 2011). También se consiguieron efectos anti-*Listeria* sinérgicos al combinar APH con distintos AEs en medio de cultivo y leche (Karatzas et al., 2001), y en zumos de frutas (Espina et al., 2013). En jamón loncheado, un tratamiento de APH (500 MPa/2 min) y aceite esencial de orégano aplicado mediante envase activo consiguió reducir *L. monocytogenes* hasta niveles inferiores a 10 UFC/g tras 20-25 días y durante todo el tiempo de análisis (70, 50 o 40 días a 4, 8 y 12 ºC, respectivamente), resultando más efectivo que el tratamiento individual de APH (Pavli et al., 2019). En jamón curado, la

combinación de APH y orizanol, o aceite de salvado de arroz, consiguió buenos resultados frente a *L. monocytogenes* (Martillanes et al., 2021).

En cuanto a los niveles de microorganismos totales presentes en el jamón, en el presente trabajo los valores de TVC fueron inferiores a 4,0 unidades logarítmicas durante 30 días cuando las muestras se almacenaron a 4 ºC, mientras que ascendieron a valores cercanos a 6,0 unidades cuando se mantuvieron en condiciones de abuso de temperatura a 12 ºC. En cualquier caso, fueron inferiores a 10^7 UFC/g, niveles que podrían relacionarse con cierto deterioro del producto. La aplicación individual de la alta presión impidió el crecimiento de TVC durante 30 días a 4 ºC, y durante 15 días a 12 ºC. El efecto de las altas presiones en los TVC ya se ha discutido en el apartado anterior. La presurización impide el crecimiento de los microorganismos totales a temperaturas de refrigeración, independientemente de la a_w. Con respecto a los bioconservantes empleados en la presente tesis doctoral, no se observaron diferencias en los TVC entre las muestras control y las tratadas individualmente con enterocinas A y B o timol. Sin embargo, cuando estos antimicrobianos se combinaron con APH a 450 MPa durante 10 min, los TVC se mantuvieron por debajo de 2,1 y 3,6 unidades logarítmicas durante 30 días a 4 y 12 ºC, respectivamente.

En cuanto a las características fisicoquímicas, se registró un aumento de pH en todas las muestras tratadas, principalmente en muestras presurizadas. Este aumento también fue observado por De Alba et al. (2013a), que atribuyó a la desnaturalización de proteínas y pérdida de protones que ocasionan las APH (Marcos et al., 2005). El timol, las enterocinas A y B, la alta presión y sus combinaciones también generaron un aumento de los valores de a_w a lo largo del almacenamiento, siendo de 0,011 unidades la mayor diferencia registrada. En general, los valores de a_w oscilaron entre 0,867 y 0,882 en muestras tratadas y control y a 4 y a 12 ºC, lo que impediría la recuperación de las células de *L. monocytogenes* dañadas pero supervivientes a los tratamientos.

El color es uno de los parámetros más importantes en la buena apariencia de los alimentos de cara a los consumidores. En este trabajo, no se registraron diferencias en el color del jamón curado entre muestras tratadas y control un día después de la aplicación de los tratamientos. Sin embargo, tras 30 días de almacenamiento y fundamentalmente a 12 ºC, se registraron alteraciones leves en algunos casos. Por ejemplo, las muestras presurizadas mostraron valores más altos de *L** (luminosidad), lo que podría deberse a cambios estructurales en las proteínas de la superficie del jamón, ya que se ha descrito que las APH inducen cambios en la luminosidad debidos a la desnaturalización de

proteínas sarcoplásmicas y miofibrilares (Angsupanich y Ledward, 1998; Marcos et al., 2010). Por el contrario, Clariana et al. (2011) observaron que la APH de hasta 600 MPa durante 6 min mantenía las características de color del jamón curado más de 50 días en refrigeración. Se ha descrito la influencia de las APH en el color rojo del jamón curado, que disminuye a medida que aumenta la intensidad de los tratamientos (Fuentes et al., 2010; Fulladosa et al., 2009), aunque el color de la carne curada es más estable frente a las APH que el de la carne fresca debido a que la nitrificación protege a la mioglobina de la oxidación inducida por alta presión (Bolumar et al., 2021). Pavli et al. (2019) tampoco registraron variaciones en el color ocasionadas por la alta presión, pero observaron un descenso en los valores de L^* y un aumento de a^* (tendencia al rojo) y b^* (tendencia al amarillo) como consecuencia de la aplicación de AEs de orégano en jamón curado. En este trabajo, también se observó un aumento en los valores de b^* en muestras tratadas con timol, pero no en a^*, que disminuyó en muestras tratadas con las enterocinas A y B, APH y tratamientos combinados. Se observó también una disminución de este parámetro en muestras de jamón curado tratadas por APH y distintas bacteriocinas (De Alba et al., 2013a).

La textura es otra de las características críticas de la calidad de un producto. En este trabajo, no se registraron diferencias como consecuencia de los tratamientos a 4 ºC, mientras que a 12 ºC, un día después de la aplicación de los tratamientos, las muestras presurizadas mostraron valores mayores de fuerza al corte. Como se ha comentado anteriormente, la APH conlleva a ruptura de uniones inter e intra-moleculares y a desnaturalización de proteínas miofibrilares y sarcoplásmicas. La reordenación posterior de dichas estructuras sería responsable del aumento de la dureza de alimentos presurizados (Clariana et al., 2011). Por el contrario, De Alba et al. (2012) registraron disminuciones en los valores de fuerza al corte al aplicar 400, 500 o 600 MPa durante 5 min en jamón curado. En cuanto al análisis sensorial, Clariana et al. (2011) concluyeron que la presurización (600 MPa/6 min) incrementó la percepción de dureza y pastosidad de jamón curado. Por otro lado, Fulladosa et al. (2009) reportaron un mayor aglutinamiento muscular así como un aumento de la dureza, la gomosidad y la fibrosidad, y una disminución de la adhesividad y la pastosidad al tratar jamón curado reestructurado con APH a 600 MPa durante 6 min. En este trabajo, los cambios en la textura ocasionados por la APH a 12 ºC se atenuaron durante el almacenamiento y, al final del mismo, ninguno de los tratamientos afectó significativamente a este parámetro a ninguna de las temperaturas ensayadas.

En definitiva, los resultados obtenidos permiten proponer la combinación de altas presiones (450 MPa/10 min) y enterocinas A y B como un método adecuado para inactivar *L. monocytogenes* en jamón curado loncheado e impedir su crecimiento durante 30 días tanto a 4 ºC como en condiciones de abuso de temperatura. Dicha combinación actuó de forma sinérgica en la reducción del patógeno, lo que posibilitó disminuir la intensidad del tratamiento de presurización, respetando las características fisicoquímicas, el color y la textura del producto.

3. Efecto de los tratamientos de inactivación en la virulencia de *L. monocytogenes*

Los tratamientos antimicrobianos pueden dar lugar a modificaciones de los mecanismos de resistencia al estrés o incluso de la capacidad de virulencia de los microorganismos supervivientes. Además de controlar el crecimiento de *L. monocytogenes* en jamón curado, un tratamiento seguro no debería afectar a la virulencia de las células supervivientes. Como se ha discutido previamente, muchos autores han investigado el uso de diversos tratamientos en el control de *L. monocytogenes* en alimentos. Sin embargo, los efectos que estos tratamientos antimicrobianos tienen en las células del patógeno supervivientes son más desconocidos. Es fundamental, por tanto, conocer el impacto en la virulencia de los patógenos tras ser sometidos a tratamientos de inactivación. Recientemente, Lucas et al. (2021) estudiaron los efectos de electrones acelerados en la expresión de genes de virulencia de *L. monocytogenes* en jamón curado concluyendo que las células supervivientes podrían ser más virulentas. En la presente tesis doctoral se investigó el efecto de las altas presiones y las enterocinas en la expresión de genes de virulencia y resistencia al estrés de *L. monocytogenes* inoculada en jamón curado.

3.1. Altas presiones en la virulencia de *L. monocytogenes*

Pese a haber mostrado buenos resultados en el control de *L. monocytogenes* en jamón curado, la inactivación por este tratamiento puede no ser completa. Como consecuencia de los pocos datos publicados al respecto, se ha considerado interesante investigar los posibles cambios en la virulencia o resistencia a situaciones de estrés de células de *L. monocytogenes* supervivientes a tratamientos de altas presiones. Jofré et al. (2007a) observaron que las proteínas de *L. monocytogenes* que se inducían durante la recuperación de bacterias dañadas tras la aplicación de APH (400 MPa/10 min) eran proteínas relacionadas con estrés y proteasas. Además de estudios de proteómica, la

transcriptómica se presenta como una buena opción para analizar alteraciones en la virulencia del patógeno. En la presente tesis doctoral, se han realizado dos trabajos independientes en los que se ha analizado la expresión de genes de virulencia y resistencia al estrés de *L. monocytogenes* en jamón curado tratado con altas presiones.

En un primer trabajo (Publicación 3.1), se inocularon lonchas de jamón curado individualmente con *L. monocytogenes* S2 (serotipo 1/2a) y S7-2 (serotipo 4b), los serotipos más frecuentes en los ámbitos alimentario y clínico, respectivamente. El producto se presurizó a 450 MPa durante 10 min o a 600 MPa durante 5 min, y se mantuvo durante 30 días a 4 ºC. El ARN se extrajo partir de las lonchas de jamón inmediatamente después de la presurización, a las 6 horas y tras 1, 7 y 30 días. Este ARN se transcribió a ADNc, y se analizó la expresión de genes de resistencia al estrés (*sigB* y *lmo1421*) y genes de virulencia (*prfA*, *plcA* y *hly*) del patógeno mediante qPCR.

Los perfiles de expresión obtenidos para cada una de las cepas fueron estadísticamente diferentes. Para la cepa S2, se observó una regulación negativa no significativa de todos los genes ensayados inmediatamente después de la presurización. Durante el almacenamiento a 4 ºC, la expresión de todos los genes, excepto el *sigB*, se incrementó, aunque las diferencias con respecto a las muestras no tratadas no fueron significativas. Por el contrario, en el caso de la cepa S7-2, la expresión de los genes *prfA*, *hly* y *sigB* aumentó significativamente en las muestras presurizadas con respecto a las muestras control, principalmente con el tratamiento a 450 MPa durante 10 min, con diferencias de incluso más de 8 unidades en el caso del *hly*. Sin embargo, durante el almacenamiento, la expresión siguió una tendencia general de regulación a la baja hasta que, tras 30 días de la presurización, todos los genes fueron reprimidos con respecto al control, sobre todo en las muestras tratadas a 600 MPa durante 5 min.

Bowman et al. (2008) aplicaron tratamientos de APH (400 o 600 MPa/5 min) a caldo de cultivo inoculado con *L. monocytogenes* y observaron una represión de la expresión de genes relacionados con virulencia, como *sigB* y *prfA*, mayor a presiones más bajas (400 MPa/5 min). De manera similar, los genes *prfA* y *sigB* también fueron reprimidos para la cepa S2 ensayada en esta tesis, inmediatamente tras la aplicación de los tratamientos, aunque fue superior a mayor presión (600 MPa/5 min). Por el contrario, los mismos genes se indujeron para la cepa S7-2 tras la presurización, siendo mayor la expresión cuando la presión aplicada fue 450 MPa durante 10 min, aunque este comportamiento se atenuó a continuación y la expresión fue reprimida a partir del día 7. Tras 30 días de almacenamiento a 4 ºC, ninguno de los genes mostró un aumento

significativo en su expresión con respecto al control sin presurizar. Estos resultados concuerdan con los obtenidos por Bowman et al. (2008), que indican que, tras ser sometida a alta presión, *L. monocytogenes* activa la transcripción de mecanismos de reparación del ADN y complejos proteicos de transcripción y traducción, entre otras cosas que ayudan a la recuperación de la bacteria, mientras que reprime genes relacionados con virulencia.

En vista de la influencia de la a_w en la efectividad de los tratamientos de APH frente a *L. monocytogenes* en jamón curado, se planteó un segundo trabajo (Publicación 3.2) para analizar su efecto en la expresión de genes de virulencia. Tras modificar la a_w de lonchas de jamón curado, se inocularon de forma individual con *L. monocytogenes* S4-2 (serotipo 1/2b) y S12-1 (serotipo 1/2c). Las muestras se presurizaron a 450 y 600 MPa durante 10 y 5 min, respectivamente y se almacenaron a 4 ºC durante un mes. Se extrajo ARN de las muestras inmediatamente tras la aplicación de los tratamientos y a los 30 días, y se utilizó para analizar la expresión del gen de resistencia al estrés *sigB* y los genes de virulencia *plcA*, *hly* e *iap*.

Al igual que en el trabajo anterior, los perfiles de expresión obtenidos para cada cepa fueron diferentes estadísticamente. Para la cepa S4-2 se registró un aumento general en la expresión de todos los genes para todos los valores de a_w del jamón presurizado, siendo dicha sobreexpresión generalmente mayor en las muestras con valores de a_w más altos y presurizadas a 450 MPa/10 min. A lo largo del almacenamiento, estas diferencias de expresión entre muestras presurizadas y control tendieron a estabilizarse, principalmente en jamón curado con a_w de 0,88 y 0,84 y sometido a 600 MPa durante 5 min. Por otro lado, todos los genes fueron reprimidos para la cepa S12-1 inmediatamente tras la presurización, sobre todo en el jamón curado con valores de a_w de 0,92 y 0,88, y siendo más pronunciada para los genes *hly* y *plcA*. Durante la refrigeración, la represión registrada inicialmente tendió a estabilizarse y, tras 30 días de almacenamiento, todos los genes continuaron reprimidos respecto a la condición control, independientemente de la intensidad de la presurización y de la a_w del jamón.

En este trabajo, la expresión del gen *sigB* de la cepa S4-2 de *L. monocytogenes*¸ que codifica un factor de resistencia a estrés general, se indujo en lonchas de jamón con distinta a_w presurizadas, siendo dicha inducción menor cuanto menor fue la a_w. Este incremento se registró también en la elaboración de embutidos curado-fermentados, durante la cual disminuyen el pH y la a_w (Mataragas et al., 2015) y también en presencia de sal (a_w = 0,96-0,97) (Olesen et al., 2010). Por el contrario, la expresión del *sigB*

disminuyó para la cepa S12-1, siendo la disminución más baja en lonchas con a_w inferior. Esta cepa es ligeramente más sensible a la APH, por lo que el daño generado en ella consecuencia de la presurización podría promover la expresión de genes de reparación de daño celular, tal y como observaron Bowman et al. (2008), como se discutió en el trabajo anterior.

3.2. Enterocinas A y B en la virulencia de *L. monocytogenes*

En un último trabajo (Publicación 3.3), se analizó el efecto de las enterocinas A y B en la expresión de genes de virulencia, principalmente de adhesión e invasión, de *L. monocytogenes* en jamón curado. Se inocularon lonchas individualmente con *L. monocytogenes* S2 (serotipo 1/2a) y S7-2 (serotipo 4b). Se añadió un extracto de enterocinas A y B en la superficie del jamón y se mantuvo durante 30 días a 4 o 20 ºC. Se extrajo ARN a partir de las lonchas de jamón inmediatamente después de la adición de las enterocinas, a las 6 horas y tras 1, 7 y 30 días y se analizó la expresión de los genes *prfA, inlA, inlB, clpC* y *fbpA*.

Una vez más, el comportamiento de las dos cepas ensayadas fue diferente. Inmediatamente después de la aplicación de las enterocinas, las diferencias de expresión génica de la cepa S2 entre las muestras tratadas y control fueron mínimas. Dicha expresión fluctuó durante el almacenamiento a ambas temperaturas, registrándose al final de los 30 días una sobreexpresión de algunos genes, mayor en muestras almacenadas a 20 ºC, y siendo solo significativa para los genes *clpC* y *fbpA*, ambos relacionados con adhesión celular. Por el contrario, en el caso de la cepa S7-2, se detectó una sobreexpresión de todos los genes en las primeras horas tras la aplicación del tratamiento, que fue más pronunciada para las muestras mantenidas a 20 ºC. Sin embargo, esta sobreexpresión inicial se atenuó a partir del día 1 y, tras 30 días, se observó una represión de los genes relacionados con adhesión e invasión, especialmente a 20 ºC. La sobreexpresión registrada para la cepa S2 al final del almacenamiento, en la mayoría de los casos no significativa, podría no suponer un incremento de la virulencia de la bacteria *in vivo* ya que esta cepa, de serotipo 1/2a, frecuentemente relacionado con aislados de origen alimentario, presenta un PMSC de tipo 6 en la secuencia de la *inlA* (Ortiz, 2016), por lo que la capacidad de invasión celular de esta cepa podría verse disminuida al tener esta proteína truncada. En el caso de la cepa S7-2, no se registraron mutaciones que generasen PMSCs en la secuencia de dicho gen (Ortiz, 2016), estando estas menos relacionadas con cepas de serotipo 4b.

Otros trabajos han mostrado cambios en la expresión por acción de bacteriocinas o cultivos bacteriocinogénicos. Así, en medio de cultivo líquido a 4 ºC, Ye et al. (2017) observaron una represión de casi todos los genes de *L. monocytogenes* ensayados en presencia de un *E. faecium* productor de bacteriocina. También Miranda et al. (2018) registraron cambios en la expresión de genes de *L. monocytogenes* relacionados con estrés cuando el patógeno se cultivó con un *Lactococcus lactis* subsp. *lactis* productor de nisina en leche desnatada reconstituida a 20 y 30 ºC durante 24 h. En jamón curado loncheado, en presencia de enterocinas o de un cultivo de *Enterococcus faecalis* productor de enterocina, Montiel et al. (2019) pusieron de manifiesto la represión de algunos genes de virulencia y resistencia a estrés de *L. monocytogenes*. Por otro lado, hay que considerar el posible desarrollo de resistencias a bacteriocinas por parte de *L. monocytogenes,* ya que Laursen et al. (2015) observaron la sobreexpresión de genes implicados en este tipo de resistencias cuando el patógeno se expuso a un sobrenadante de *Lactobacillus plantarum* con pediocina.

Con respecto a la investigación sobre la expresión génica, es necesario considerar en primer lugar que la expresión de genes de *L. monocytogenes* podría variar tan solo por el hecho de encontrarse en una matriz alimentaria con unas características intrínsecas determinadas como es el jamón curado. Se ha demostrado que las concentraciones de NaCl que se dan comúnmente en jamón curado pueden incrementar la actividad de genes relacionados con estrés, como *sigB*, y producen la sobreexpresión de *lmo1421*, que es un gen de tolerancia a estrés general y dependiente de SigB (Liu et al., 2014; Mataragas et al., 2015; Olesen et al., 2010; Sue et al., 2003). Sin embargo, en los trabajos de transcriptómica realizados en esta tesis, la expresión génica se analizó normalizando frente a un gen de referencia y se relativizó con respecto a una condición control (jamón curado no presurizado o no tratado con enterocinas A y B), por lo que las características del producto que pueden influir en la expresión del patógeno (a_w, NaCl, nitritos, etc.) no han sido consideradas, ya que el objetivo fue investigar exclusivamente el efecto de los tratamientos de presurización y bioconservación.

La falta de información respecto al efecto de tratamientos de inactivación de *L. monocytogenes* en matrices alimentarias reales, las diferentes condiciones de ensayo, así como las diferentes cepas y genes del patógeno analizados, hacen complicado establecer comparaciones entre los resultados obtenidos en esta tesis y los datos recogidos en la bibliografía. En los tres trabajos de transcriptómica realizados, se comprobó que los

tratamientos influyeron en los patrones de expresión de las células de *L. monocytogenes* supervivientes y que dichos efectos fueron específicos de cepa. También se han observado diferencias dependientes de cepa en la expresión génica tras la exposición del patógeno a distintas concentraciones de sal en un medio a base de queso (Schrama et al., 2013), en un modelo de jamón curado con distinta a_w y contenido de NaCl a distintas temperaturas (Alía et al., 2019), en patés de hígado con distinto contenido en sal (Olesen et al., 2010), tras su exposición a un choque térmico moderado (Wałecka-Zacharska et al., 2018), o al utilizar un extracto de enterocinas o un cultivo de *E. faecalis* productor de bacteriocina para controlar *L. monocytogenes* en jamón curado (Montiel et al., 2019). Estas diferencias podrían deberse a muchos factores, como el serotipo, aunque un comportamiento específico de cepa en los patrones de expresión y la virulencia potencial se ha registrado también en cepas de *L. monocytogenes* del mismo serotipo sometidas a estrés ácido y osmótico (Olesen et al., 2009).

En los tres trabajos realizados en esta tesis, los niveles de expresión génica fluctuaron a lo largo del almacenamiento independientemente de la cepa de *L. monocytogenes*, de los genes ensayados o del tratamiento aplicado. Así, aunque en algunos casos se registró una sobreexpresión de los genes de virulencia inmediatamente después de la aplicación de los tratamientos o en las primeras horas, la expresión de dichos genes tendió a atenuarse, registrándose al final del almacenamiento incluso una represión de genes inicialmente inducidos. También en jamón curado, Montiel et al. (2019) comprobaron cambios en la expresión génica de *L. monocytogenes* a lo largo del almacenamiento cuando se trató con enterocinas A y B o con un cultivo de *E. faecalis* productor de bacteriocinas, al igual que Lucas et al. (2021), que observaron una fluctuación a lo largo del tiempo que finalizó con un aumento de la expresión de los genes de virulencia del patógeno cuando se sometió a un tratamiento de electrones acelerados de 3 kGy.

La expresión de genes de virulencia y resistencia a estrés también resultó modificada por la temperatura de conservación en leche ultrapasteurizada, embutidos fermentados, queso blando y carne picada (Rantsiou et al., 2012). En la publicación 3.3 de esta tesis se observaron resultados similares. La cepa de *L. monocytogenes* serotipo 1/2a tendió a incrementar la expresión de genes de virulencia, principalmente relacionados con adhesión e invasión, tras 30 días de almacenamiento del jamón curado en condiciones de abuso de temperatura (20 ºC) respecto a temperaturas de refrigeración (4 ºC). De forma similar, Duodu et al. (2010) concluyeron que los niveles de transcritos

del gen *inlA* de una cepa considerada poco virulenta fueron significativamente más altos en salmón mantenido a 20 ºC que a 4º C.

El factor SigB contribuye a la osmorregulación y a la tolerancia a ácidos, bilis y antimicrobianos que actúan sobre la pared celular (Begley et al., 2006; Sue et al., 2003; Wemekamp-Kamphuis et al., 2004; Zhang et al., 2011), así como frente a tratamientos de procesado de alimentos como APH (Wemekamp-Kamphuis et al., 2004). En la Publicación 3.2 de esta tesis sobre el impacto de la a_w en jamón curado presurizado, la tendencia en los patrones de expresión de los genes *plcA* y *hly* fueron similares a la tendencia registrada para el gen *sigB* en cada una de las cepas, lo que concuerda con el hecho de que SigB controle también la síntesis de PrfA, el principal regulador de la virulencia de *L. monocytogenes,* que regula a su vez la expresión de genes de virulencia como *hly*, *plcA*, *inlA* o *inlB*, entre otros. Los promotores de *hly* y *plcA* presentan una simetría perfecta y gran afinidad por PrfA, siendo considerablemente sensibles a dicho factor. Por tanto, una mayor expresión de *prfA* conlleva a una mayor concentración de PrfA, lo que podría promover una mayor expresión de *hly* y *plcA*. En la Publicación 3.1, en la que se analizó el efecto de la APH en la expresión génica del patógeno, la tendencia de estos dos genes de virulencia para cada cepa ensayada fue similar a la observada para *prfA*, siendo más marcada en el caso del gen *hly*, lo que podría justificarse con la existencia de promotores independientes de PrfA para dicho gen. Algo similar se observó en los resultados obtenidos en la Publicación 3.3, con una represión del gen *prfA* para las dos cepas ensayadas inmediatamente después de la aplicación del tratamiento con enterocinas A y B frente a la inducción observada para los genes *inlA* e *inlB*, lo que puede deberse de nuevo a la presencia de promotores adicionales para estos genes. De hecho, al final del almacenamiento, los patrones de expresión de las internalinas A y B para la cepa S7-2 fueron similares al patrón del gen *clpC*, que codifica una ATPasa que regula también la expresión de ambas internalinas y es necesaria para los procesos de adhesión e invasión (Nair et al., 2000).

Los resultados obtenidos en esta tesis doctoral indican que tratamientos de altas presiones y la aplicación de enterocinas A y B para el control de *L. monocytogenes* en jamón curado alteran los patrones de expresión de genes de resistencia a estrés y virulencia del patógeno. No obstante, en ninguno de los casos se ha podido establecer una relación entre dichos cambios de expresión y una mayor virulencia o resistencia a estrés atribuible al tratamiento. En este sentido, sería necesario profundizar en esta investigación. Además, debería analizarse el efecto de un tratamiento combinado de altas

presiones (450 MPa/10 min) y enterocinas A y B en la expresión de genes de virulencia y resistencia a estrés de *L. monocytogenes*, ya que este ha sido el tratamiento combinado más eficaz de los ensayados en esta tesis, ejerciendo un efecto anti-*Listeria* sinérgico y sin modificar apenas las características del jamón curado. Asimismo, sería muy interesante investigar el efecto de los tratamientos, individuales y/o combinados, en la capacidad de adhesión de *L. monocytogenes* supervivientes a líneas celulares intestinales humanas, con el fin de complementar los resultados obtenidos y establecer una correlación entre los datos de transcriptómica y estudios *in vivo* para alcanzar conclusiones más sólidas respecto a la virulencia de las cepas.

Conclusiones

De los resultados obtenidos en esta tesis doctoral se han extraído las siguientes conclusiones:

1. *L. monocytogenes* se aísla en superficies limpias de la zona de deshuesado de empresas productoras de jamón curado, con presencia mayoritaria de complejos clonales hipovirulentos adaptados al ambiente de la industria. La detección de cepas persistentes señala la necesidad de mejorar las medidas de control. La caracterización genética de estas cepas permitiría estimar el riesgo potencial en caso de producirse contaminación.
2. La aplicación de un tratamiento de altas presiones a 600 MPa durante 5 min permite inactivar *L. monocytogenes* en el interior de jamones deshuesados enteros. Su eficacia disminuye en la superficie del jamón debido a la mayor concentración de sal y menor actividad de agua, aunque estas características impiden el crecimiento del patógeno en el producto.
3. En jamón curado loncheado, la actividad de agua baja ejerce un efecto baroprotector sobre *L. monocytogenes*, disminuyendo la eficacia de las altas presiones.
4. Las enterocinas A y B son más eficaces que el timol en la inactivación de *L. monocytogenes* en jamón curado loncheado. Sin embargo, su aplicación no evita la recuperación del patógeno durante el almacenamiento.
5. La aplicación de enterocinas A y B permite disminuir la intensidad del tratamiento de altas presiones a 450 MPa durante 10 min, consiguiendo un efecto anti-*Listeria* sinérgico que, además, impide la recuperación del patógeno durante el almacenamiento del jamón curado loncheado.
6. Las características fisicoquímicas, el color y la textura del jamón curado no resultan apenas afectados por los tratamientos de altas presiones y los bioconservantes aplicados individualmente o en combinación.
7. Los tratamientos de altas presiones en jamón curado loncheado con distinta actividad de agua y las enterocinas A y B inducen cambios en los patrones de expresión de genes de virulencia y resistencia a estrés de *L. monocytogenes* que son específicos de cepa. En su conjunto, los estudios de transcriptómica no permiten concluir una mayor virulencia potencial en las células supervivientes a los tratamientos.

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